



Digitized by the Internet Archive
in 2010 with funding from
University of Toronto

THE JOURNAL OF
EXPERIMENTAL MEDICINE

J.

70

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY
SIMON FLEXNER, M.D.

VOLUME THIRTIETH
WITH FIFTY-SIX PLATES AND EIGHTY-ONE
FIGURES IN THE TEXT



154808
-90/4/20

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1919

COPYRIGHT, 1919, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

R
11
J68
v. 30
cop. 3

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

✓

CONTENTS.

No. 1, JULY 1, 1919.

	PAGE
NOGUCHI, HIDEYO. Etiology of yellow fever. IV. The acquired immunity of guinea pigs against <i>Leptospira icteroides</i> after the inoculation of blood of yellow fever patients.....	1
NOGUCHI, HIDEYO. Etiology of yellow fever. V. Properties of blood serum of yellow fever patients in relation to <i>Leptospira icteroides</i>	9
NOGUCHI, HIDEYO. Etiology of yellow fever. VI. Cultivation, morphology, virulence, and biological properties of <i>Leptospira icteroides</i> . Plates 1 to 3.....	13
KLIGLER, I. J. Growth accessory substances for pathogenic bacteria in animal tissues.....	31
KAWAMURA, K. Studies on organ transplantation. I. Transplantation of the thyroid gland with intact blood supply. Plates 4 to 6.....	45
KAWAMURA, K. Studies on organ transplantation. II. Transplantation of the spleen with intact blood supply. Plates 7 and 8.....	65
HARRIS, WILLIAM H., and CHILLINGWORTH, FELIX P. The experimental production in dogs of emphysema with associated asthmatic syndrome by means of an intratracheal ball valve. Plates 9 to 11.....	75

No. 2, AUGUST 1, 1919.

NOGUCHI, HIDEYO. Etiology of yellow fever. VII. Demonstration of <i>Leptospira icteroides</i> in the blood, tissues, and urine of yellow fever patients and of animals experimentally infected with the organism.....	87
NOGUCHI, HIDEYO. Etiology of yellow fever. VIII. Presence of a leptospira in wild animals in Guayaquil and its relation to <i>Leptospira icterohamorrhagiæ</i> and <i>Leptospira icteroides</i> ...	95

DRAGSTEDT, LESTER R., DRAGSTEDT, CARL A., MCCLINTOCK, JOHN T., and CHASE, C. S. Intestinal obstruction. II. A study of the factors involved in the production and absorption of toxic materials from the intestine.....	109
CLOUGH, MILDRED C. A study of pneumococci reacting with antipneumococcus sera of Types I, II, and III, with an observation of a mutation of one of the strains.....	123
LUNDGAARD, CHRISTEN. Studies of oxygen in the venous blood. V. Determinations on patients with anemia.....	147
JONES, F. S. The streptococci of equines. Plate 12.....	159

No. 3, SEPTEMBER 1, 1919.

DOCHEZ, A. R., AVERY, O. T., and LANCEFIELD, R. C. Studies on the biology of streptococcus. I. Antigenic relationships between strains of <i>Streptococcus hæmolyticus</i>	179
STADIE, WILLIAM C. The oxygen of the arterial and venous blood in pneumonia and its relation to cyanosis. Plates 13 to 15.....	215
HARROP, GEORGE A., JR. The oxygen and carbon dioxide content of arterial and of venous blood in normal individuals and in patients with anemia and heart disease.....	241
LUNDGAARD, CHRISTEN. Studies on cyanosis. I. Primary causes of cyanosis.....	259
LUNDGAARD, CHRISTEN. Studies on cyanosis. II. Secondary causes of cyanosis.....	271
LUNDGAARD, CHRISTEN. Erythrosis, or false cyanosis.....	295

No. 4, OCTOBER 1, 1919.

SMITH, THEOBALD, and TAYLOR, MARIAN S. Some morphological and biological characters of the spirilla (<i>Vibrio fetus</i> , n. sp.) associated with disease of the fetal membranes in cattle. Plate 16.....	299
SMITH, THEOBALD. The etiological relation of spirilla (<i>Vibrio fetus</i>) to bovine abortion.....	313
SMITH, THEOBALD. The bacteriology of bovine abortion, with special reference to acquired immunity.....	325

SMILLIE, ERNEST W., LITTLE, RALPH B., and FLORENCE, LAURA. An interpretation of the agglutination reaction to <i>Bacillus abortus</i> in 75 cases of bovine abortion bacteriologically controlled.....	341
AVERY, O. T., and CULLEN, GLENN E. Hydrogen ion concentration of cultures of pneumococci of the different types in carbohydrate media.....	359
LORD, FREDERICK T. The relation of proteolytic enzymes in the pneumonic lung to hydrogen ion concentration. An explanation of resolution.....	379
LORD, FREDERICK T., and NYE, ROBERT N. The relation of the pneumococcus to hydrogen ion concentration, acid death-point, and dissolution of the organism.....	389
NOGUCHI, HIDEYO. Etiology of yellow fever. IX. Mosquitoes in relation to yellow fever.....	401

No. 5, NOVEMBER 1, 1919.

JACOBS, WALTER A., and HEIDELBERGER, MICHAEL. Chemotherapy of trypanosome and spirochete infections. Chemical series. I. <i>N</i> -Phenylglycineamide- <i>p</i> -arsonic acid.....	411
BROWN, WADE H., and PEARCE, LOUISE. Chemotherapy of trypanosome and spirochete infections. Biological series. I. The toxic action of <i>N</i> -phenylglycineamide- <i>p</i> -arsonic acid.	417
PEARCE, LOUISE, and BROWN, WADE H. Chemotherapy of trypanosome and spirochete infections. Biological series. II. The therapeutic action of <i>N</i> -phenylglycineamide- <i>p</i> -arsonic acid in experimental trypanosomiasis of mice, rats, and guinea pigs.....	437
PEARCE, LOUISE, and BROWN, WADE H. Chemotherapy of trypanosome and spirochete infections. Biological series. III. The therapeutic action of <i>N</i> -phenylglycineamide- <i>p</i> -arsonic acid in experimental trypanosomiasis of rabbits. Plates 17 to 37.....	455
BROWN, WADE H., and PEARCE, LOUISE. Chemotherapy of trypanosome and spirochete infections. Biological series. IV. The action of <i>N</i> -phenylglycineamide- <i>p</i> -arsonic acid upon spirochete infections. Plates 38 to 44.....	483

WINCHELL, AGNES I., and STILLMAN, ERNEST G. The occurrence of <i>Bacillus influenzae</i> in the normal throat.....	497
MILLS, RALPH G. The pathological changes in the testes in epidemic pneumonia. Plates 45 to 50.....	505

No. 6, DECEMBER 1, 1919.

EBELING, ALBERT H. A strain of connective tissue seven years old. Plates 51 to 55	531
STEVENS, F. A., and KOSER, S. A. Streptolysin production in carbohydrate media.....	539
WOLLSTEIN, MARTHA. Pfeiffer's bacillus and influenza. A serological study.....	555
BARBER, M. A. A study by the single cell method of the influence of homologous antipneumococcic serum on the growth rate of pneumococcus.....	569
BARBER, M. A. Antiblastic phenomena in active acquired immunity and in natural immunity to pneumococcus.....	589
LANGSTROTH, LOVELL. Blood viscosity. I. Conditions affecting the viscosity of blood after withdrawal from the body..	597
LANGSTROTH, LOVELL. Blood viscosity. II. Effect of increased venous pressure.....	607
HOGUE, MARY JANE. The effect of hypotonic and hypertonic solutions on fibroblasts of the embryonic chick heart <i>in vitro</i> . Plate 56.....	617
INDEX TO VOLUME XXX.....	649

ETIOLOGY OF YELLOW FEVER.

IV. THE ACQUIRED IMMUNITY OF GUINEA PIGS AGAINST LEPTOSPIRA ICTEROIDES AFTER THE INOCULATION OF BLOOD OF YELLOW FEVER PATIENTS.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 2, 1919.)

Most of the attempts to transmit yellow fever by inoculating the blood of patients into various animals have ended in failure. As stated elsewhere¹ out of 74 guinea pigs, in only eight were the results positive, and in other species of animals they were all negative. It should be emphasized that for the purpose of transmission cases were selected which were still in the early stage of the disease, previous investigators having pointed out the fact that the virus may no longer exist in the peripheral blood after the 3rd day of the illness.

In the present study the guinea pigs which failed to manifest the typical symptoms, or any symptoms at all, were kept under daily observation for many weeks. On a later date, through temporary shortage of normal guinea pigs, they were inoculated, together with several normal guinea pigs, with a virulent organ emulsion, rich in the leptospira, from a guinea pig experimentally infected with the organism, in order to prepare a large amount of organ emulsion for the purpose of immunizing two donkeys against the virus. A great many of the guinea pigs remained perfectly well, while most of the normal animals, as well as those which had been previously inoculated with the blood of malarial patients,² came down with an experimental infection.

¹ Noguchi, H., *J. Exp. Med.*, 1919, xxix, 565.

² In a country where aestivo-autumnal parasites infest the population many times a malarial patient is brought to the Yellow Fever Hospital on the chance that the case is one of early yellow fever. All these cases were used for the experiments on the transmission of yellow fever without the loss of time which would have been caused by waiting for a diagnosis, for which a day or two might be required.

TABLE I.
Susceptibility of Guinea Pigs to the Inoculation of Leptospira icteroides 25 Days after They Had Been Injected with the Blood of Yellow Fever Patients.

Patient.	Sex.	No. of guinea pig.	Day of disease when bled.	Amount of blood injected.	Reaction.	Subsequent test for susceptibility.	Reaction.	Result.
Case 27. Yellow fever. Died in 16 days.	M.	16	14th	2	None.	Virulent Arias strain after 25 days.	40.3 in 5 days.	Killed in 8 days. Typical.
		17	14th	3	"	" "	39.8 " 6	Died in 8 days. Typical.
Case 25. Yellow fever. Mild. Recovery. Also malaria.	F.	21	6th	3	"	" "	39.9 " 6	Died in 8 days. Typical.
		22	6th	3	39.7 in 4 days.	" "	None.	Remained well.
Case 1. Malaria (Guayaquilian).	"	23		3	None.	" "	40.5 in 8 days.	Killed in 11 days. Mild but typical.
		24		4	"	" "	40.0 " 7	Killed in 9 days. Typical.
Case 27. Yellow fever. Second specimen of blood.	M.	32	15th	5	"	" "	39.9 " 5 Icterus.	Killed in 7 days. Typical.
		33	15th	5	"	" "	40.0 in 6 days. Icterus.	Died in 8 days. Typical.
Case 13. Dr. Valenzuela's patient. Yellow fever. Mild. Recovery.	"	35	3rd	3	39.8 in 8 days.	" "	40.2 in 4 days.	Recovered.
		36	3rd	5	39.2 " 12	" "	39.6 " 5	Remained well.

Case 2. Malaria.	M.	30		2	None.	Virulent Arias strain after 25 days.	40.4 in 4 days. Icterus.	Died in 9 days. Typical.
" 3. "	"	52		3	"	"	39.9 in 6 days. Icterus.	Died in 10 days. Typical.
" 4. "	"	19		2	"	"	40.0 in 9 days. Icterus.	Killed in 12 days. Typical.
" 7. Yellow fever. Severe. Recovery.	"	20		2(?)	"	"	40.0 in 11 days. Slight icterus.	Died in 17 days. Mild but typical.
" 7. Yellow fever. Severe. Recovery.	"	37	6th	5	39.8 in 6 days.	"	None.	Remained well.
Case 12. Yellow fever. Moderate. Recovery.	F.	39	2nd	5	39.2 " 8 "	"	39.0-39.7 in 5-6 days.	"
Case 5. Malaria.	"	40	2nd	3	39.2 " 8 "	"	None.	"
" 27. Yellow fever. Autopsy material 6 hrs. post mortem.	"	41		3	39.6 " 9 "	"	40.0-40.3 in 9 days.	Died in 13 days. Typical.
		42		5	39.7 " 6 "	"	40.5 in 7 days. Icterus.	Died in 10 days. Typical.
	M.	48	16th	2 (kidney emulsion).	None.	"	40.0 in 8 days. Jaundice later.	Recovered (!).
		49	16th	"	"	"	40.0 in 7 days. Slight jaundice later.	"
Case 21. Yellow fever. Died on 4th day.	"	54 55	4th 4th	3* 3	39.4 in 5 days. 39.8 " 5 "	" "	None. "	Remained well. "

*Another guinea pig (No. 56) received the same amount at the same time and died in 7 days with suspicious lesions. From it transfers were made into two guinea pigs. Both showed mild fever in 5 days and recovered. The two also resisted the subsequent inoculations of virulent Arias strain.

TABLE I—*Concluded.*

Patient.	Sex.	No. of guinea pig.	Day of disease when bled.	Amount of blood injected.	Reaction.	Subsequent test for susceptibility.	Reaction.	Result.
Case 22. Yellow fever. Died in 10 days.	M.	198	5th(?)	3	°C. 40.0 in 4 days. Trace of icterus (?).	Virulent Arias strain after 25 days. “ “	None. °C.	Remained well.
		199	5th	3	40.0 in 7 days. Trace of icterus (?).		“	“
Case 19. Yellow fever. Severe. Recovery.	“	200	4th(?)	3	39.6 in 4 days.	“ “	“	“
		201	4th	3	40.0 “ 5 “ Trace of icterus.		“	“
Case 20. Yellow fever. Severe. Recovery.	“	220	4th(?)	3	40.3 in 7 days. Trace of icterus (?).	“ “	“	“
		221	4th	3	40.2 in 5 days. Trace of icterus (?).		“	“
Case 16. Yellow fever. Severe. Recovery.	“	222	3rd(?)	3	40.1 in 7 days. Trace of icterus (?).	“ “	“	“
		223	3rd	3	40.3 in 6 days. Trace of icterus (?).		“	“

Case 28. Yellow fever. Died.	M.	329	(?) Brought in unconscious. Died same day.	5	None.	Virulent Arias strain after 25 days.	40.0 in 5 days. Icterus.	Died in 7 days. Typical.
Case 10. Yellow fever. Died in 7 days.	F.	338	2nd	4	"	"	40.1 in 6 days. Icterus.	Killed in 8 days. Typical.
		339	2nd	4	"	"	39.5 in 7 days. Icterus.	Killed in 10 days. Typical.
Case 24. Yellow fever. Died.	M.	340	(?) Died next day.	4	39.2 in 5 days. No icterus. Died in 8 days; hemorrhages.			
		341	" "	4	40.0 in 13 days. No icterus.	"	40.0 in 6 days. Icterus.	Died in 9 days. Typical.
Case 8. Yellow fever. Severe. Recovery.	"	342	2nd	4	40.0 in 12 days. No icterus.	"	40.2 in 7 days. Icterus. None.	Died in 10 days. Typical.
		343	2nd	4	39.6 in 6 days. Trace of icterus.	"		Remained well.
Case 18. Yellow fever. Died in 7 days.	"	407	4th	3	39.8 in 5 days. Trace of icterus.	"	"	"

Apparently some of the guinea pigs previously inoculated with the blood from yellow fever cases were refractory to the subsequent inoculation with a known virulent organ emulsion. As shown in Table I, those which resisted the infection were found to have had a febrile reaction of varying degree and duration on about the 5th day after the injection of the blood. This seemingly unimportant fever reaction must have been the result of a very mild abortive infection totally unsuspected on account of the absence of the main symptom, the jaundice. This fact is proved, in my opinion, by the acquired resistance of an animal which, when a sufficient quantity of a passage strain of *Leptospira icteroides* is introduced, is so susceptible. There is reason to believe that the strains of *Leptospira icteroides* as they occur in man are on the whole less virulent to the guinea pig and are incapable of producing a fatal infection from the beginning except in rare instances. It may be recalled here that with one strain (Case 2) the organism did not become thoroughly adapted to the guinea pig until it had been passed through three generations in this animal.¹

A complete, or nearly complete refractoriness or resistance to *Leptospira icteroides* was demonstrated in sixteen guinea pigs previously inoculated with the blood of yellow fever patients, representing nine out of fourteen cases of yellow fever. Four out of six guinea pigs injected with the blood of two other yellow fever patients died, and two survived. In this group the protection was present in some of the guinea pigs only. In a doubtful case one of the two guinea pigs resisted a subsequent infection. On the other hand, of six guinea pigs, which received the blood of three patients, all died with the typical symptoms and lesions when tested later with a virulent passage strain of *Leptospira icteroides*. Of ten, which received injections of blood from four malarial patients, all proved to be susceptible to a later inoculation with the same strain of *Leptospira icteroides* that was used in the foregoing experiments.

Of the guinea pigs representing the yellow fever group, those which had had a febrile reaction several days after the injection of the blood are the ones which acquired the immunity against the inoculation of *Leptospira icteroides*. There seems to exist some relation between the febrile reaction and the acquired immunity. In several instances, moreover, these guinea pigs showed a suspicious trace of

jaundice in the scleras some time after the height of fever had passed, but the disease had failed to advance further. If the animals had been killed at the proper time and transfers made to normal guinea pigs the virulence of the causative organism might have been gradually increased to reproduce the disease more completely in later passages. As a matter of fact it was possible to accomplish this in one instance (Case 2).

The injection of blood from some cases of typical yellow fever into guinea pigs caused a rise of temperature in about 5 days similar to that of other cases, and there was even a suspicion of a trace of icterus in the scleras, yet no protection against a later infection with a passage strain of *Leptospira icteroides* (Case 1) could be demonstrated. Perhaps this lack of protection may be explained by assuming the strain variations among different strains of the leptospira.

Another point of interest brought out in this series is that the injection of organ emulsions obtained from fresh postmortem material (Case 27) conferred upon guinea pigs sufficient protection against a subsequent infection to prevent its being fatal, although these animals showed fever and jaundice. It is possible that the emulsion contained a certain amount of the antigen and produced a mild immunity, or a limited amount of the immune bodies.

SUMMARY.

The majority of guinea pigs inoculated with the blood of yellow fever patients escaped a fatal infection.

There were a number of instances in which the inoculation of yellow fever blood induced in these animals a temporary febrile reaction on the 4th or 5th day, followed in some cases by slight jaundice, but with a rapid return to normal. Most of these guinea pigs when later inoculated with an organ emulsion of a passage strain of *Leptospira icteroides* resisted the infection. On the other hand, the animals which had previously been inoculated with the blood of malaria patients or normal guinea pigs died of the typical experimental infection after being inoculated with the infectious organ emulsion.

It appears from the results just described that a number of non-fatal, mild, or abortive infections follow the inoculation of blood of yellow fever patients into guinea pigs. The fact that such animals manifested refractoriness to a subsequent attempt to infect with a highly virulent passage strain of *Leptospira icteroides* is an indication, judging from the reciprocal immunity reaction, that they actually passed through an infection with the same organism, or a strain closely related to it, as that which was used for the second infection experiment

ETIOLOGY OF YELLOW FEVER.

V. PROPERTIES OF BLOOD SERUM OF YELLOW FEVER PATIENTS IN RELATION TO *LEPTOSPIRA ICTEROIDES*.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 2, 1919.)

Following the isolation of *Leptospira icteroides* from a case of yellow fever a series of experiments was instituted with a view to establishing the relation between the organism and the disease. As stated elsewhere,¹ the organism reproduces in experimental animals all the symptoms and lesions observed in man. From the standpoint of immunity the question arose whether or not the serum of yellow fever convalescents would have a specific influence upon the organism.

Blood was drawn from the median basilic vein of the patient and the serum mixed with *Leptospira icteroides* in the form of organ emulsion from infected guinea pigs or culture and injected into the peritoneal cavity of a normal guinea pig. After a period of 30 minutes to 1 hour the fluid was drawn from the peritoneal cavity by means of a sterile capillary pipette and examined under the dark-field microscope (Pfeiffer's phenomenon). The animals were allowed to live until the results of the inoculations were evident. As Table I shows, in some of the experiments, in which the amount of the infectious emulsion or culture used was too large, no certain protection of the animal from the final fatal infection was obtained with the serum of convalescents, notwithstanding the fact that such serum produced a definite Pfeiffer phenomenon in the peritoneal cavity. When a smaller quantity of the infecting material was used in combination with convalescent serum most of the animals were saved, while the controls with serum from patients not suffering from

¹ Noguchi, H., *J. Exp. Med.*, 1919, **xxix**, 585.

Effect of Serum from Yellow Fever Patients upon *Leptospira icteroides*.

Case No.	Sex.	Age.	Severity of infection.	Day on which serum was taken.	Amount of serum.		Lep- to- spi- ra- icte- roides. Strain 1.		Pfeiffer phenomenon	Result.
							Liver emul- sion.	Culture.		
		yrs.			cc.	cc.	cc.			
9	M.	19	Mild. Recovered.	2nd	1	0.2		-	Died in 8 days.	
9	"	19	" "	10th	1	2		+	Survived.	
2	"	23	" "	12th	2	2		+	"	
14	"	23	Severe. "	10th	2	2		<+	Died in 7 days.	
Control.	"	21	Malaria.		2	2		-	" " 7 "	
"	"	20	"		2	2		-	" " 7 "	
"					Saline, 1 cc.	2		-	" " 7 "	
"					Saline, 2 cc.	2		-	" " 6 "	
7	M.	21	Severe. Recovered.	16th	1	0.1		+	Survived.	
12	F.	40	" "	11th	2	0.1		+	"	
9	M.		Diagnosis (?). Re- covered.	30th	2	0.1		-	Died in 6 days.	
Control.					Ringer, 2 cc.	0.1		-	" " 10 "	
"					Ringer, 2 cc.	0.1		-	" " 9 "	
16	M.	25	Severe. Recovered.	19th	1		1	<+	All these sera	
26	"	21	" "	18th	2		1	-	were sepa-	
29	F.	34	Mild. "	13th	1		1	-	rated from	
19	M.	25	Severe. "	24th	2		1	+	the clot in 48	
30	"	21	Mild. "	14th	2		1	+	hrs. and then	
31	"	22	Moderate. "	20th	1		1	+	kept on ice for	
23	"	16	" "	22nd	2		1	+	2 to 4 wks.	
32	"	20	Mild. "	15th	2		1	+	when they	
33	"	11	" "	7th	2		1	+	were tested.	
8	"	21	Severe. "	14th	2		1	+	The negative	
20	"	20	Moderate. " (also malaria).	13th	2		1	<+	results with the sera of Cases 26 and 29 may be due to deteriora- tion through age.	
34	F.	30	Mild. Recovered.	12th	2		1	+		

yellow fever, and with saline solution died with the typical symptoms.

The number of cases studied (eighteen) was limited, owing to the pressure of more urgent problems under investigation at the same time. It seems, however, to have been sufficient to establish the specific reaction which exists between *Leptospira icteroides* and the serum of yellow fever convalescents (fifteen positive, 83 per cent). In the case of one patient the serum did not have any effect upon the organism when tested on the 2nd day of the illness but was protective on the 10th day.

Sera from malarial patients in no case showed any action antagonistic to *Leptospira icteroides*. The malarial patients were all mountaineers and consequently non-immune to yellow fever. The negative results obtained with sera derived from doubtful cases of yellow fever, all so mild as to make it difficult to recognize the disease, might have been due to the absence of antibodies, or, if there were any, to the fact that they were too weak to produce a definite reaction under the experimental conditions, or, a not impossible assumption, to the existence of a variation among many strains.

As will be described later, ten normal sera from healthy, non-immune soldiers² were also examined before the vaccination of the latter with killed cultures of *Leptospira icteroides*, but none of them had any effect upon the organism.

SUMMARY.

The serum from a number of persons recovering from yellow fever in Guayaquil was studied with a view to establishing its possible immunological relationship with a strain of *Leptospira icteroides* derived from one of the yellow fever patients. For this purpose the serum of convalescents was mixed either with an organ emulsion of a passage strain, or with a culture of the organism, and inoculated intraperitoneally into guinea pigs.

² I am indebted for these specimens to Dr. Carlos A. Miño, Assistant Director of the Department of Health at Quito, to Dr. E. Salgado V., also of the Department of Health, and to Dr. Víctor M. Bayas, Surgeon of the "Bolivar" Regiment.

The Pfeiffer reaction was first studied, and then the animals were allowed to live until the controls, inoculated with the same emulsion or culture of *Leptospira icteroides* but without the serum, or with serum from patients suffering from other diseases than yellow fever, had died of the experimental infection with typical symptoms. A positive Pfeiffer phenomenon was observed in fifteen of the eighteen convalescent cases studied, or approximately 83 per cent. Sera from ten non-immune soldiers and from two malaria patients gave uniformly negative results. Protection from an ultimate fatal infection was afforded some of the guinea pigs which received the serum of yellow fever convalescents, while the control animals succumbed to the infection with typical symptoms. In one instance, in which the serum was tested on the 2nd and the 10th days of disease, a Pfeiffer reaction was demonstrated, as well as protective property against the infection, in the specimen from the 10th but not in that from the 2nd day.

From the foregoing observations of immunity reactions it appears highly probable that *Leptospira icteroides* is etiologically related to yellow fever.

ETIOLOGY OF YELLOW FEVER.

VI. CULTIVATION, MORPHOLOGY, VIRULENCE, AND BIOLOGICAL PROPERTIES OF *LEPTOSPIRA ICTEROIDES*.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 1 TO 3.

(Received for publication, May 2, 1919.)

Cultivation.

As the nature of the causative agent of yellow fever was unknown, it was necessary at the beginning of these experiments to formulate a special method of cultivation. The methods employed have been similar to those recommended¹ for the cultivation of *Leptospira icterohæmorrhagiæ* (Inada and Ido). Instead of the serum and citrate plasma of the rabbit or other animal, serum and plasma from non-immune persons were used during the early stage of the cultivation experiments. The principal medium consisted of a mixture of 1 part of the serum and 3 parts of Ringer solution, used in a combination of the liquid form and a form made semisolid by adding melted neutral agar (0.3 per cent), the liquid half (8 cc.) of the medium being superimposed on the semisolid half (8 cc.) in a tall culture tube such as that used in the cultivation of spirochetes.²

The first step in the inoculation of the medium was to mix about 0.5 to 1 cc. of the citrate blood, drawn from the median basilic vein of the patient, with the lower or semisolid portion of the medium, while the latter was still in the fluid state (42°C.), and allow the mixture to solidify by cooling. The serum-Ringer dilution was then poured on the semisolid portion and about 0.5 to 1 cc. more of the same blood introduced. A layer of paraffin oil was finally added to

¹ Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 575.

² Noguchi, H., *J. Exp. Med.*, 1912, xvi, 199.

cover the surface of the medium. When making a culture with the citrate blood from a patient no human citrate plasma was added to the liquid portion of the medium, as the plasma contained in the blood was sufficient to form a loose fibrin throughout that portion. When subsequent subcultures were set up, however, 0.5 to 1 cc. of citrate human or rabbit plasma was introduced into the liquid portion after inoculation. The presence of a loose cobweb fibrin in the culture medium seems to favor the growth of certain organisms.¹ The conditions provided for in this form of culture medium would allow the growth of various microorganisms requiring different degrees of oxygen tension. In a later period 2 to 3 cc. of the citrate blood were used for each of two or three large flasks (50 cc.) with correspondingly larger quantities of culture medium (25 cc.).

Direct Cultivation from Yellow Fever Patients.

Cultures were made from eleven cases of yellow fever, with only three successful isolations of the leptospira. In the first instance (Case 1) I failed to detect the organisms under the dark-field microscope, but in a culture 3 days old (kept at 26°C.) a few active leptospiras were seen. This was inoculated into four guinea pigs, all of whom died later of the typical experimental infection. One of the guinea pigs had epistaxis and melena in addition to intense jaundice, advanced degeneration of the liver, acute parenchymatous nephritis, ecchymoses in the lungs, stomach, and intestines.³ This culture was presently lost through a secondary fungus contamination, which was difficult to avoid under the conditions in which the work had to be carried on.

The second positive growth was obtained with the blood derived from Case 4 on the 3rd day of the disease. The organisms were readily detected in the culture after 5 days at 30°C. A few leptospiras were present in the blood when carefully examined in the stained preparations, and the guinea pigs inoculated with this specimen came down with the typical symptoms in 8 days. The culture proved to be pathogenic for guinea pigs.

³ Noguchi, H., *J. Exp. Med.*, 1919, xxix, 565.

The third successful direct cultivation of the organism from yellow fever patients was obtained with the blood from a fatal case (Case 6). The blood was drawn on the 5th day of the disease and put immediately into six tubes containing the culture medium (October 19, 1918). On October 26 one of the inoculated tubes showed the presence of the leptospira under the dark-field microscope. The culture proved to be capable of producing the typical symptoms and lesions in guinea pigs, pups, and marmosets.⁴

Cultivation from Experimental Animals.

The method employed for obtaining a culture of *Leptospira icteroides* was the same as that outlined for direct cultivation from human blood, except that normal rabbit serum and citrate plasma were used instead of human. The blood was obtained from the heart before the death of the animal. The method was not always successful in the first generation, but it was nevertheless the most reliable of the various combinations tried. In a later subculture the addition of the citrate plasma becomes unessential, although a better growth is had when it is added.

Six strains (Cases 1 to 6) of yellow fever leptospira have been maintained to date by passage in guinea pigs. No direct culture was obtained with the blood from Cases 2, 3, and 5, although cultures were finally obtained from the blood of guinea pigs or marmosets inoculated with the passage strain. These cultures, whether obtained directly from the blood of yellow fever patients or indirectly by way of animal inoculation, were found to be uniform in their characteristics and could be maintained in culture for many months. The leptospira isolated from yellow fever cases is extremely sensitive to any alien microbic intrusion, not surviving the slightest contamination in culture, and the failure to obtain a culture directly from yellow fever cases can in every instance be explained through the occurrence of secondary contamination.

When the guinea pigs reach the stage of collapse, with intense jaundice, it is seldom possible to detect the leptospira in the circulation, and a positive transfer to normal guinea pigs becomes uncertain.

⁴ Noguchi, H., *J. Exp. Med.*, 1919, xxix, 585.

Cultures set up with this blood usually remain sterile, and the leptospira is not found in the liver and kidneys. In experimental infectious jaundice, on the contrary, the leptospira was almost always found in the later stage of the infection. The extreme lability of *Leptospira icteroides* may account for the negative animal inoculation and microscopic findings in so many cases of yellow fever.

Morphology.

The organism which occurs in the blood and tissues in yellow fever patients in Guayaquil, as well as in those of animals experimentally infected with the blood or tissue of yellow fever patients, is an extremely delicate filament measuring about 4 to 9 microns in length and 0.2 of a micron in width along the middle portion. It tapers gradually toward the extremities, which end in immeasurably thin sharp points. The entire filament is not smooth but is minutely wound at short and regular intervals, the length of each section measuring about 0.25 of a micron. The windings are so placed as to form a zigzag line by the alternate change of direction of each consecutive portion at an angle of 90° .

The organism is unrecognizable by translucent light but becomes quite visible under a properly adjusted dark-field illumination. It possesses an active motility, consisting in vibration, rotation, rapid bipolar progression, and sometimes twisting of parts of the filament. When it encounters a semisolid substance it penetrates the latter by a boring motion, and while passing through it the body assumes a serpentine aspect with few undulations, the elementary windings undergoing no modification.

The organism manifests remarkable flexibility to almost any angle while changing its course of progression in a semisolid medium. In a fluid medium it has fewer and quite characteristic movements. One end is usually bent in the form of a graceful hook, and, while rapidly rotating, the organism proceeds in the direction of the straight end, the hooked end apparently serving as a sort of rear propeller. When extricating itself from an entanglement, however, the same hooked end seems to act like the front propeller of an airplane. Many specimens are seen with both ends hooked, the organism then ro-

tating in a stationary position unless one hook is larger and more powerful as a propeller than the other. The rapid rotation makes the organism appear like a chain of minute dots. From the dynamic point of view the portions which include the several windings from the extremities represent the motor apparatus of the organism. I have never seen a specimen that doubled at the middle portion of the body while lying in a free liquid medium. The motor or terminal portions may be regarded as comparable with the flagella or terminal filaments seen in a spironema or treponema.

The organism is difficult to stain with ordinary aniline dyes, but can be made distinct by osmic acid fixation and one of the Romanowsky stains (Giemsa, Wright, Leishman). When stained with Fontana or carbolized gentian violet solution after mordanting with 5 per cent tannin plus 1 per cent phenol the organism appears as a moderately heavy, slightly undulated filament without a clear elementary indentation. The peculiar forms resembling the letters C and S are quite characteristic. Specimens fixed with methyl alcohol seldom retain the elementary spirals. The beauty of the organism as it appears by dark-field illumination is never well retained in a stained preparation, even in the best specimens. In the latter it appears almost as a totally different organism.

From the findings described it is evident that the present organism falls in the general order of so called spirochetes, but in the strict sense of the term it is neither a bacterium, a spirochete, a spironema, nor a treponema, but belongs to the genus *Leptospira*, of which *Leptospira icterohæmorrhagiæ*, *Leptospira hebdomadis*,⁵ and *Leptospira biflexa* have already been described.¹

The study of the strains of *Leptospira icteroides* obtained from yellow fever cases in Guayaquil showed the organisms to be of somewhat smaller dimensions than the various strains of *Leptospira icterohæmorrhagiæ* in my possession (six strains), as is readily seen from the photographs of the organisms shown in Figs. 1 to 9. The difference is striking when the pictures of the two organisms are compared, particularly in the case of Strain 6 of *Leptospira icteroides*, which is considerably smaller than any of the other strains.

⁵ Ido, Y., Ito, H., and Wani, H., *J. Exp. Med.*, 1918, xxviii, 435.

The strains of *Leptospira icterohæmorrhagiæ* isolated from wild rats caught in Guayaquil are also shown (Figs. 10 to 13). These are seen to be similar to the other strains of *Leptospira icterohæmorrhagiæ*, shown in Figs. 5 to 9, and are coarser than the organism obtained from yellow fever cases.

The photographs shown in the three plates were taken for the purpose of comparison at the same time, under similar conditions, and with the same magnification.

Cultural Properties.

Leptospira icteroides does not multiply in a medium in which there is no access to oxygen. In a dense solid medium it grows well within the zone or layer to which a trace of oxygen can still penetrate, but no deeper. It grows best when the supply of oxygen is not excessive, as when a thin layer of liquid paraffin is poured over the surface of the culture medium. A certain amount (above 10 per cent) of a suitable blood serum is essential for its growth. Various bacterial culture substances such as peptone, meat extract, various carbohydrates in different forms, or combinations, are unsuitable; their presence in the serum-containing media apparently neither favors nor impedes development. The percentage of sodium chloride (tried as high as 2 per cent) in the medium seems to have but little influence, and either isotonic saline, Ringer solution, or distilled water may be used as diluent. The organism is highly sensitive to the reaction of the medium, the optimum growth being obtained with a reaction slightly alkaline to litmus paper, not stronger than 0.025 N. It grows well in a neutral medium, but not in one with an acid reaction to litmus paper.

The addition of phenol red to culture media in a ratio of 1 cc. of a 0.0025 per cent solution to 10 cc. of medium has no perceptible disturbing effect upon the growth of *Leptospira icteroides*. Growth takes place in culture tubes in which phenol red indicates the values ranging from pH 6 to pH 7.4. In the case of cultures containing rabbit serum phenol red is gradually decolorized to a trace of pink.

Growth is much more rapid at a temperature of 37°C. than at 25–26°C., but the organisms remain viable much longer at the latter

temperature. No growth is obtainable at a temperature above 42°C . or below 10°C .

Erythrocytes present in the culture do not undergo any special alteration that can be ascribed to the growth of this organism, nor does the hemoglobin. The serum proteins seem in no wise modified and remain transparent. No external changes of the culture media, except a light layer of grayish haze over the surface of a solid or semisolid medium observed in richly growing old cultures, take place, and for this reason the growth of the organism can be ascertained microscopically only.

It has been noticed that *Leptospira icteroides* shows a particular preference for a semisolid medium such as is provided by the presence of agar (0.3 per cent) or by loose fibrin. They entangle themselves in the substance in large numbers and move about in it very actively. Continuous multiplication goes on in this type of medium. After a few weeks the growth may become so dense as to render the uppermost layer of the medium faintly grayish. This peculiarity of the organism may partly account for its predilection for the parenchymatous organs such as the liver and kidney.

Leptospira icteroides multiplies through transverse fission.

Virulence.

While the pathogenic properties of *Leptospira icteroides* for different species of animals have not been exhaustively studied, it has been shown that most of the domestic animals, such as the donkey, horse, sheep, pig, and cat are completely refractory to the injection of the organism. Very young dogs, not older than 6 or 7 weeks, are found to succumb to experimental infection. None of the birds so far employed for experiment has been found to be susceptible.³ Among the mammals the guinea pig appears to be most susceptible and the marmoset somewhat less so. For this reason the guinea pig has been chosen for determining the degree of virulence of several strains of *Leptospira icteroides*.

The mode of inoculation consisted in intraperitoneal injection in descending doses of a culture 2 to 3 weeks old. The guinea pigs used varied from 300 to 350 gm. A 2 to 3 weeks old culture (26°C .) of this organism, grown in a semisolid rabbit serum medium with

0.15 per cent agar, may contain 50 to 100 leptospiras per field (Leitz $\frac{1}{12}$ oil immersion and ocular 4). In order to arrive at an accurate determination of virulence different strains would have to be used in correspondingly comparable concentrations. But this is extremely difficult in the case of an organism which forms entangled masses of many individuals or shows a tendency to congregate in varying numbers about the particles of culture medium. In the present series of experiments suspensions of cultures of different strains were so prepared as to make each contain approximately an equal number of organisms in the suspension from which higher dilutions were prepared. Each strain was used in successive tenfold dilutions, and one or two guinea pigs were inoculated with 1 cc. each of each dilution. Because

TABLE I.
Determination of Virulence of Leptospira icteroides, Strain 1.

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	days	
1	1	3	Died in 7 days.
2	0.1	4	Survived (!). No jaundice.
3	0.01	5	Died in 9 days.
4	0.001	3½	" " 8 "
5	0.0001	4	" " 9 "
6	0.00001	5	" " 10 "
7	0.000001	No fever.	Survived.

of individual variations in resistance among the guinea pigs in all later experiments two animals were used for each dilution.

Four strains of *Leptospira icteroides* were studied in this way. The results are recorded in the following protocols.

Experiment 1.—Aug. 10, 1918 (at the Guayaquil Yellow Fever Hospital) Strain 1. 18 day culture of the second generation, grown on semisolid human serum agar at 30°C. (Table I.)

Experiment 2.—Dec. 2, 1918. Strain 3. 20 day culture of the third generation, grown on semisolid rabbit serum agar medium at 26°C. (Table II.)

Experiment 3.—Jan. 2, 1919. Strain 5. 3 week culture of the third generation, isolated from a marmoset experimentally infected with a visceral emulsion from a guinea pig which died of the typical infection after inoculation with blood from a fatal yellow fever case. The culture was grown on the same medium as that used for Strain 6, at 26°C. (Table III.)

TABLE II.

Determination of Virulence of Leptospira icteroides, Strain 3.

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	days	
1	1	3½	Died in 8 days.
2	1	5	Survived (!).
3	0.1	4	Died in 9 days.
4	0.1	5	" " 8½ "
5	0.01	5	Survived (!).
6	0.01	5½	Died in 11 days.
7	0.001	4	" " 9 "
8	0.001	5	Survived.
9	0.0001	5	"
10	0.0001	No fever.	"
11	0.00001	7	"
12	0.00001	No fever.	"
13	0.000001	" "	"

TABLE III.

Determination of Virulence of Leptospira icteroides, Strain 5.

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	days	
1	1	3	Died in 7 days.
2	0.1	3	" " 8 "
3	0.1	2½	" " 6 "
4	0.01	3½	" " 7 "
5	0.01	4½	" " 9 "
6	0.001	5	" " 10 "
7	0.001	3½	" " 8 "
8	0.0001	5	" " 10 "
9	0.0001	4	" " 9 "
10	0.00001	6	Survived.
11	0.00001	No fever.	"
12	0.000001	" "	"
13	0.000001	" "	"

Experiment 4.—Jan. 2, 1919. Strain 6. 20 day culture of the third generation, directly derived from human blood (not passed through guinea pigs), grown on semisolid rabbit serum agar at 26°C. There were about 25 organisms per field in the original suspension. (Table IV.)

The foregoing experiments show that the strains of *Leptospira icteroides* possess, on the whole, a strong virulence for guinea pigs. In two cases (Nos. 1 and 6) the minimal lethal dose was 0.00001 cc., in one (No. 5) 0.0001 cc., and in another (No. 3) 0.001 cc. But in the experiments with Strains 1 and 3 some of the guinea pigs receiving as large a quantity as 1 cc. or 0.1 cc., showed only a transient febrile reaction and speedily returned to normal, notwithstanding the fact that 0.0001 part of these doses killed other guinea pigs in the same series of experiments. This is not altogether exceptional,

TABLE IV.

Determination of Virulence of Leptospira icteroides, Strain 6.

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	days	
1	1	4	Died in 7 days.
2	0.1	3	" " 10 "
3	0.1	3½	" " 6 "
4	0.01	4	" " 8 "
5	0.01	5 (?)	" " 7 "
6	0.001	3	" " 9½ "
7	0.001	5	" " 10 "
8	0.0001	4	" " 10 "
9	0.0001	Doubtful.	Survived.
10	0.00001	3	Died in 8 days.
11	0.00001	6	Survived.
12	0.000001	4	"
13	0.000001	No fever.	"

because in the higher dilutions there were instances in which a smaller dose induced a fatal infection while a larger one failed to do so. Again, in fatal instances the severity of the infection did not parallel the amount of culture injected. In other words, the susceptibility of guinea pigs varies considerably among different individuals. In another series of experiments, not yet reported, it was noticed that certain guinea pigs possess an almost complete natural immunity to *Leptospira icteroides*. This becomes an important factor in a consideration of the percentage of successful transmissions of this organism from human cases to guinea pigs.

Gradual Loss of Virulence through Cultivation.

All the strains of *Leptospira icteroides* were brought to New York from Guayaquil on semisolid rabbit serum agar. The cultures were kept at ordinary temperature during the journey (about 28°C. in the tropics and 15°C. after reaching the United States). They had been renewed in Guayaquil on October 26, 1918, and were tested for their pathogenicity for guinea pigs in New York on December 2, 1918; that is, 37 days after the transfer into new media. Intraperitoneal inoculations were made into guinea pigs of 1 cc. of Strains 1, 3, 4, 5, and 6, and the animals developed the usual symptoms and lesions characteristic of the infection produced by these strains, showing that under the circumstances described the organism remained virulent for 37 days.

On December 9, 1918, some of the older cultures of Strains 1 and 3, which had stood over 4 months since cultivation, were also tested, with varying success. Strain 1 was still quite virulent, but Strain 3 failed to produce a fatal infection. In a subsequent experiment, however, by using six guinea pigs, each being inoculated with 2 cc. of the culture intraperitoneally, it was possible to obtain a fatal infection in one of the animals. Through this guinea pig the virulence of the culture was again raised to its original height; that is, it again became capable of causing typical infection in guinea pigs in smaller quantities.

It should be noted that examination of the viscera, especially the lungs, of the guinea pigs which escaped death or severe infection from the inoculation of an attenuated strain, by killing them at the end of about 14 days from the time of inoculation, usually revealed the presence of old hemorrhages of greater or less extent in the lungs. Perhaps it may prove a useful procedure for ascertaining the outcome of transmission to inoculate several guinea pigs with the blood of a yellow fever patient and examine the lungs within a period of from 10 to 14 days. In this way, notwithstanding the absence of striking external manifestations, the results of inoculation can be more accurately followed. Unfortunately this fact was not known at the time of the experiments, reliance being placed upon the development of a fatal infection.

For the past 4 months horse serum has been used for culture media, since it was easily obtainable at much less expense and in larger quantities than rabbit or sheep serum. It is far less satisfactory, however, for the cultivation of *Leptospira icteroides*, than sheep serum, which in turn is much inferior to rabbit serum. In testing out the virulence of different strains of the organism recently, rather rapid loss of virulence has been encountered. The reason was not at first clear, the period of time being comparatively short, but it soon became evident that cultivation of *Leptospira icteroides* on sheep or horse serum media leads to rapid diminution of virulence, since the cultures grown on rabbit serum media remained pathogenic. By following the course of development of these strains in media containing rabbit serum and phenol red it was found that the original pinkish color of the indicator gradually becomes paler until it fades to a trace. The color may be brought back to a deeper hue by the addition of disodium phosphates, but it never reaches the original grade. Apparently a change takes place in the media as well as in the indicator through the growth of the organism. Such a change has not been observed in the cultures grown on media containing sheep or horse serum. Whether the persistence of virulence of the organism in the media containing the rabbit serum has any relation to the phenomenon just described or whether they are two unrelated coincidental phenomena has not been further studied.

Resistance and Viability.

Leptospira icteroides is a non-spore-bearing organism and offers little resistance to the action of heat, desiccation, putrefaction, or disinfectants.

Heating to 55°C. for 10 minutes or freezing and thawing kill the organism, and complete desiccation promptly destroys its vitality.

In the presence of various bacteria, such as *Bacillus coli*, *Bacillus aerogenes*, *Bacillus subtilis*, *Bacillus mesentericus*, *Bacillus pyocyaneus*, pneumococcus, staphylococcus, *Streptococcus hemolyticus*, etc., *Leptospira icteroides* is destroyed within a short time. The more numerous the bacteria the quicker the disappearance of the leptospira; hence in decomposing excreta or urine, sewer or stagnant water, or in con-

taminated foodstuffs, no leptospira can be found 24 hours after being introduced into them.

On the other hand, certain contaminating fungi or non-putrefactive and non-acid-producing bacteria, sometimes bacilli and sometimes cocci, have been found growing in the cultures of *Leptospira icteroides* without seriously interfering with the viability of the latter, which are actively motile among the intruding fungi or bacteria. In such a contaminated culture these intruders do not cause any perceptible modification of the culture medium, except that their discrete colonies may be found imbedded here and there in the medium.

When a pure culture of the organism was poured into a cup of sterile distilled water and left unprotected from the air or dust the leptospires survived several days, but finally disappeared, partly because of the lack of nutrition and partly because of bacterial growth. The leptospira intentionally added in large quantity to fecal matter kept at room temperature disappeared within a few hours.

On several occasions attempts were made to infect the larvæ of *Stegomyia calopus* by introducing emulsions of liver or kidney containing a large number of the organisms into the receptacle with the larvæ, but no leptospira could be found in such a mixture after 2 hours. In this respect the virus of yellow fever is one of the least resistant of all pathogenic organisms which have been obtained in culture. In my experience there has seldom been an impure culture of this organism.

Another interesting phenomenon in connection with the organism in question is that it soon dies out; it may degenerate within 12 hours in a piece of liver or kidney removed from an infected guinea pig and kept at a temperature of about 10°C. In hundreds of instances a leptospira was found only rarely in the liver, kidney, or blood from guinea pigs which had died of typical experimental yellow fever several hours before autopsy. In this respect *Leptospira icteroides* differs considerably from *Leptospira icterohæmorrhagiæ* isolated from the cases of infectious jaundice in Japan or Europe, the latter being still easily recoverable from animals kept over night after death.

With regard to the resistance of the organism to the action of various ordinary disinfectants the work is still incomplete. It has been found, however, that it is readily killed within 5 minutes by 2 per

cent phenol or 0.1 per cent bichloride of mercury. In a 10 per cent solution of sodium taurocholate, sodium glycocholate, or sodium cholate the organism promptly disintegrates, but saponin has no injurious effect upon it. Human or animal bile dissolves the organism rapidly when used in concentrations stronger than 30 per cent.

Filterability.

A noteworthy characteristic of *Leptospira icteroides* is its ability to pass through the pores of filters. Some experiments to determine this point were carried out as early in the investigation as the transmission of Strain 1 from the human case to guinea pigs. By the use of Berkefeld filters V and N with suction by means of a water pump it was possible to filter an emulsion of the liver and kidney of a guinea pig experimentally infected with the passage strain 7 days previously. The clear filtrates, which were bacteriologically sterile, were inoculated intraperitoneally into normal guinea pigs in doses of 10 cc. each on August 8, 1918. Both animals came down with typical symptoms after $7\frac{1}{2}$ and 8 days respectively. In the blood of these animals a small number of leptospiras were demonstrated 24 hours before death. In the emulsions of the liver and kidney the organisms were also present, and upon further passage to normal guinea pigs the emulsion proved to be infectious.

Possibility of the Existence of a Granular Phase in the Life of Leptospira icteroides.

That there may exist a granular phase of life in various members of the family of spirochetes has been repeatedly suggested by investigators. Balfour, Fantham, Leishman, and Todd⁶ advanced the idea that the spirochetes of relapsing fevers in man and fowls pass through a granular stage at some time in their life. The following observation seems strongly to suggest the possibility that this phenomenon also occurs in the life of *Leptospira icteroides*.

⁶ Balfour, A., *Internat. Congr. Med.*, 1913, xxi, 275. Fantham, H. B., *Ann. Trop. Med. and Parasit.*, 1914, viii, 471. Leishman, W. B., *Internat. Congr. Med.*, 1913, xxi, 282. Todd, J. L., personal communication.

The various cultures of *Leptospira icteroides* made at Guayaquil on October 26, 1918, were brought back to New York on November 24 without special accident. But on examination no leptospira could be found in any of the tubes containing cultures of Strain 5, although the other cultures were growing well. A thorough examination of the eleven tubes of Strain 5 was continued for several days without success.

Six culture tubes which were made on October 18, 1918, with the blood from Marmoset 4, severely infected with Strain 5, and which had been showing a fairly good growth on October 26, were also examined. These tubes showed no spiral organisms. There were large numbers of refringent granules imbedded in the culture medium in which the leptospiras had been abundantly present a month previously. These granules appeared to be the degenerated remains of the leptospiras. The hope of recovering the strain from these cultures was almost abandoned, but as a last resort a dozen guinea pigs were inoculated with 1 cc. of the contents of each of these tubes. Some of these animals in due time came down with typical symptoms. The spiral forms of the leptospiras were found in varying numbers in the blood, liver, and kidneys of these animals, and a culture of the strain was regained.

It is of course possible that these old culture tubes contained the spiral leptospira in such small numbers that they escaped microscopic detection, but it is also possible that they existed in a granular phase under certain conditions.

SUMMARY.

By the employment of methods designed to promote the growth both of aerobic and anaerobic organisms, particularly those belonging to the class of spirochetes, it was possible to obtain a pure culture of a delicate organism, the morphological features of which place it in the genus *Leptospira*. On three occasions, that is, from three out of eleven cases of yellow fever, the organism was directly cultivated. These three strains were found to induce the characteristic symptoms and lesions when tested on guinea pigs. The organism was designated *Leptospira icteroides*.

Leptospira icteroides was also obtained in pure culture from the blood of guinea pigs which succumbed to infection after being inoculated with the blood or organ emulsions from patients suffering from yellow fever. These cultures also proved to be virulent when tested on susceptible animals.

The morphological characteristics and certain biological properties of the organism were considered in detail. It is invisible under translucent illumination and is difficult to stain by most aniline dyes. It is highly sensitive to the presence of bacteria and is rapidly destroyed in a medium in which certain other organisms are present. The presence of blood serum (man, sheep, horse, rabbit, etc.) seems to be essential for its growth. It grows well at a temperature of about 25–26°C. and more quickly at 37°C., though at the latter temperature it dies out within a few weeks. At 25°C. under favorable conditions and in suitable culture media it remains viable for several months without losing its virulence. *Leptospira icteroides* multiplies by transverse division.

The virulence attained by some strains was such that 0.00001 cc. of a culture could induce typical fatal infection in guinea pigs. There exists a considerable variation among guinea pigs in their susceptibility to *Leptospira icteroides*.

The organism is killed within 10 minutes at a temperature of 55°C. and is also destroyed by complete desiccation or freezing and thawing. Bile and bile salts dissolve it in certain concentrations, but not saponin.

Leptospira icteroides passes through the pores of Berkefeld filters V and N, and there is a possibility of its having a granular phase of life under certain conditions.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Dark-field view of a 2 week culture on semisolid rabbit serum agar medium of *Leptospira icteroides*. Strain 6 (Case 6). $\times 3,000$.

FIG. 2. The same. Strain 4 (Case 4).

FIG. 3. The same. Strain 5 (Case 5).

FIG. 4. The same. Strain 3 (Case 3).

PLATE 2.

FIG. 5. Dark-field view of a 2 week culture on semisolid rabbit serum agar medium of *Leptospira icterohæmorrhagæ*. Japanese strain. $\times 3,000$.

FIG. 6. The same. British strain.

FIG. 7. The same. French strain.

FIG. 8. The same. American Strain 1.

FIG. 9. The same. American Strain 2.

PLATE 3.

FIG. 10. Dark-field view of a 2 week culture on semisolid rabbit serum agar medium of *Leptospira icterohæmorrhagæ*. Group 8 strain obtained from wild rats in Guayaquil. $\times 3,000$.

FIG. 11. The same. Group 11 strain.

FIG. 12. The same. Group 30 strain.

FIG. 13. The same. Group 30 strain.



(Noguchi: Etiology of yellow fever. VI.)



(Noguchi: Etiology of yellow fever. VI.)



GROWTH ACCESSORY SUBSTANCES FOR PATHOGENIC BACTERIA IN ANIMAL TISSUES.

By I. J. KLIGLER, PH. D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 8, 1919.)

The subject of vitamins has of late occupied the attention of the biologist and biological chemist. The nature of these substances is still a matter of speculation, but the majority of workers accepts the distinction proposed by McCollum and Davis. These authors recognize two classes of substances, the fat-soluble A and the water-soluble B. A more precise definition has not been possible, because of the failure thus far to isolate and identify the respective compounds. Their presence can be detected only by the effect they produce on the growing organism, usually the white rat. By noting the effect of the addition of various substances to a balanced vitamin-free diet, one can obtain a rough indication of the concentration of food accessory substances in the added material.

The subject of the relation of vitamins to bacteria has recently been approached from two directions. One group of workers studied the bacteria with a view to discovering the source of vitamins. Since plants (Bottomley) and animals (McCollum and Simmonds, Osborne and Mendel, Loeb and Northrop) cannot synthesize these substances, their origin must naturally be looked for among the lower microorganisms. Mockenridge has indeed shown that certain soil bacteria are capable of elaborating these substances, although *Bacillus radicumicola*, actively synthetic and of simple food requirements, cannot do so. Pacini and Russell report experiments indicating that the typhoid bacillus grown in Uschinsky's medium produces vitamins. The other group of workers was more concerned in determining the relation of vitamins to the cultivation of the more delicate pathogenic bacteria. Cole and Lloyd demonstrated their importance for the growth of the gonococcus, while Lloyd and Gordon, Hine, and

Flack showed that they were essential also for the growth of the meningococcus. Evidently neither of the organisms can multiply actively in the absence of these substances.

The object of this paper is to report experiments bearing on (a) the effect of vitamins on the growth of a number of organisms pathogenic for man; (b) the distribution of these substances in animal tissues; and (c) the relative significance of the fat-soluble A and water-soluble B in the cultivation of these microorganisms. At present there are few data bearing on these questions in relation to bacteria.

EXPERIMENTAL.

Methods.

In the course of the investigation beef heart, goat blood, rabbit and cat tissues, and human secretions were used. Unless otherwise stated, the method of extraction was always the same. The tissue or organ was obtained as free from blood as possible. The animals were first exsanguinated and the tissues were washed with saline solution, free from visible traces of blood. The material was then weighed, macerated into small bits, suspended in nine times its weight of saline solution, shaken thoroughly, and placed in the ice box over night. The following day the extract was centrifugalized and filtered through a Berkefeld candle. After testing for sterility the extracts were ready for use.

The effect of these extracts was tested by adding graded amounts to nutrient broth or agar, or to phosphate peptone agar and inoculating with small amounts of culture suspensions. The tubes were incubated at 37°C. and observations were made daily for several days. Control tubes without extract were always used.

The following organisms were tested:¹ gonococcus, meningococcus (one para, one regular), Pneumococcus Type I, *Streptococcus hæmolyticus*, *Bacillus diphtheriæ*, *Bacillus pertussis*, *Bacillus influenzae*.

¹ Cultures of *B. typhosus* and of the Shiga and Flexner varieties of *B. dysenteriae* were included in the early experiments, but as no differences were observed between the growth on the control media and on those containing the extracts, they were later excluded.

To avoid the error that would result from carrying over vitamins with the bacterial mass, small inocula were used. Usually 0.1 cc. of a 24 hour broth culture was suspended into 5 cc. of peptone or saline solution, and of this suspension 0.1 cc. was used for inoculating broth tubes and a 4 mm. loop for the agar slants. Before making the suspensions the cultures were first diluted to a turbidity corresponding to a 24 hour broth culture of the pneumococcus.

Results.

Most of the experiments reported below were repeated from one to four times. Either broth or agar media or both were used. As a rule (when the size of the inoculum was just right), the effect of the extract was manifested both by the earlier appearance and greater abundance of the growth. The extracts, with few exceptions, influenced favorably the growth of all the bacteria studied, and the effect varied with the amount of extract added.

Effect of Beef Heart Extract.—The following is a typical experiment with beef heart extract. The extract was prepared from fresh beef heart in the manner outlined, and varying amounts were added to tubes containing 5 cc. of nutrient broth. The results are shown in Table I. Similar results were obtained with the use of agar as a base.

TABLE I.
Effect of Heart Extract on the Growth of Bacteria.

Culture.	Amount of extract.									
	1.0 cc.		0.1 cc.		0.01 cc.		0.001 cc.		0.0 cc.	
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.
Meningococcus.....	+	++	+	++	—	±	—	—	—	—
Pneumococcus.....	++	++	+	++	+	+	—	+	—	+
Streptococcus.....	++	++	—	+	—	—	—	—	—	—
<i>B. diphtheria</i>	±	+++	—	+++	—	++	—	+	—	+
<i>B. pertussis</i>	++	++	++	++	—	+	—	+	—	+
<i>B. influenza</i> †.....	—	+	—	+	—	—	—	—	—	—

* The plus signs indicate the presence and relative abundance of growth.

† Inoculations of the influenza bacillus were made with a 4 mm. loop direct from blood broth cultures; small amounts of blood were thus carried over, but not enough to permit growth in plain broth.

Effect of Extracts of Various Organs and Tissues of the Rabbit and Cat.—A uniform procedure was employed in the following experiments. The animal was etherized, exsanguinated, and the organs or tissues were removed aseptically to sterile Petri dishes. The extracts were made as described. With stomach or intestinal mucosa the organs were first washed free of fecal matter, the mucosa was then scraped off with a scalpel and suspended in nine times its weight of saline solution. Extracts from tissues of four rabbits and one cat were tested in the course of the experiments. The method of testing was the same as that described above.

The results were strikingly uniform. When 1 cc. of the extracts was added, no differences in effect were discernible. Extracts of all the tissues tested had a decidedly stimulating action on the growth of the various test organisms. However, when smaller amounts of the extract were added, certain fairly constant differences were observed. Extracts of liver and stomach mucosa were more effective than those of other tissues, while those of brain and muscle were less so. The influence of other tissue extracts was practically the same. They had somewhat different effects on different bacteria, but that may have been due to the error in technique which is considerable.

Table II gives the results obtained in one series of tests. It is difficult to summarize the data. Attention can only be called to some of the points which cannot be tabulated. The differences between the tubes containing larger and those containing smaller amounts of the extract were always more marked after 24 than after 48 hours. Not only were the colonies on the slants of the former more numerous, but they were, as a rule, larger. The pneumococcus showed a strikingly different behavior on spleen extract agar than on the others. On the former medium autolysis usually occurred 24 to 48 hours after it had appeared in all the other tubes. *Bacillus influenzae* failed to grow on any of the media until a trace of blood was added. When a drop of a saline suspension of red cells was added to the slant, the growth was practically the same in all the tubes. The results with the gonococci were extremely variable. Even under favorable conditions growth is much slower than that of any of the organisms tested. As a rule, 3 to 4 days elapse before fair sized colonies appear. By that time the tubes are apt to be dry,

and moisture is essential for their growth. They did, however, grow as well on agar containing 0.1 cc. of the extracts as on sheep serum glucose agar.

TABLE II.

Effect of Extracts of Rabbit Tissues on the Growth of Bacteria.

Culture.	Amount of extract.	Extract.								
		Liver.	Spleen.	Kidney.	Heart.	Lung.	Muscle.	Brain.	Testes.	Stomach.
	cc.									
Streptococcus....	0.1	++	++	++	+	+	+	+	+	++
	0.01	=	=	=	=	=	=	=	=	+
	0.0	=	=							
Pneumococcus....	0.1	+++	+++	+++	+++	++	++	++	+++	+++
	0.01	++	++	++	++	++	++	++	++	++
	0.0	+	+							
<i>B. diphtheriæ</i>	0.1	++	++	++	++	++	+	=	=	++
	0.01	+	=	++	=	=	=	Tr.	=	++
	0.0	—	—							
Gonococcus.....	0.1	+	+	++	+	+	+	+	+	++
	0.01	+	+	++	+	+	+	+	+	++
	0.0	—	—							
Meningococcus...	0.1	++	++	++	++	++	+	=	++	+++
	0.01	++	+	+	++	++	+	Tr.	++	+++
	0.0	—	—							

Growth Accessory Substances in Extract of Mucosa of Various Organs.

—The interesting fact brought out by the previous experiments was that extract of stomach mucosa stimulated the growth of bacteria more actively than those of other tissues. It was of interest to note whether extracts of other mucosa would have a similar effect. It was also anticipated that these extracts might show some specific selective action. Tests were made with nasopharyngeal, stomach, intestine, and genitourinary mucosa. The usual method of extraction and testing was employed. The results showed clearly that extracts of all the mucosa tested had a favorable effect on growth. No

conclusion could be drawn from these experiments regarding their specificity, because of the large element of error involved in the technique.

Growth Accessory Substances in Cat Tissues.—The object of these tests was to determine whether there was any marked difference in the distribution of the growth accessory substances in different animals. The extracts were made and tested in the usual manner. The only difference in technique was the use of peptone phosphate agar and peptone phosphate broth in place of the beef infusion medium. The former furnished a vitamine-free base, and the effects of the extracts were brought out more strikingly than when infusion media

TABLE III.

Effect of Extracts of Cat Tissues on the Growth of Bacteria.

Culture.	Control.	Extract (0.1 cc. to tube).							
		Liver.	Spleen.	Kidney.	Heart.	Lung.	Brain.	Stomach.	Intestines.
<i>Streptococcus</i>	—	++	++	++	++	++	+	++	++
<i>Pneumococcus</i>	—	++	++	++	++	+	++	++	++
<i>B. diphtheriæ</i>	—	++	++	++	++	++	++	++	++
<i>Meningococcus</i>	—	++	++	++	++	++	++	+++	++
<i>B. pertussis</i>	—	+	+	#	+	#	#	#	+

were used. Otherwise the results were essentially the same as those obtained with rabbit tissue extracts. As was previously noted, the autolysis of the pneumococcus occurred later on liver and spleen extract media than on the others. The results are shown in Table III.

Effect of Tissue Extracts on Lag.—The phenomenon of lag is of practical as well as theoretical interest. It has been studied extensively by a number of workers, among them Müller, Penfold, and Chesney. The most carefully worked out theory explaining this phenomenon is that advanced by Chesney. He believes that lag represents the time necessary for the bacterial cells to recover from injury.

It seemed of interest to determine the effect of the extracts on the lag phase, or, in other words, the influence of growth-stimulating substances on the speed of cell recuperation. Beef heart and rabbit tissue extracts were used. The streptococcus and pneumococcus served as the test cultures, because of the ease with which they can

TABLE IV.
Effect of Beef Heart Extract on Bacterial Lag.

Test culture, <i>Streptococcus hemolyticus</i> .				
Platings.	Time interval. <i>hrs.</i>	No. of colonies per cc. of broth.		
		Extract, 1 cc.	Extract, 0.1 cc.	Broth.
1	0	2,300	3,300	1,300
2	1	3,800	3,900	2,500
3	3	32,000	11,000	1,400
4	5	230,000	14,000	>100
5	6	320,000	43,000	>100
	24	Heavy turbidity.	Sterile.	Sterile.

TABLE V.
Effect of Beef Heart Extract on Bacterial Lag.

Test culture, pneumococcus.				
Platings.	Time interval. <i>hrs.</i>	No. of colonies per cc. of broth.		
		Extract, 1 cc.	Extract 0.1 cc.	Broth.
1	0	3,200	3,000	3,200
2	1	3,700	3,200	4,000
3	3	4,000	4,000	4,100
4	5	4,600	4,300	1,300
5	6	15,000	8,000	>1,000
	24	Good turbidity.	Sterile.	Sterile.

be manipulated. To tubes containing 5 cc. of broth were added graded amounts of the extract. Duplicate tubes of broth and extract broth were inoculated with the same amount of a culture suspension. Glucose agar plates were made immediately and at stated

intervals. The plates were incubated at 37°C. for 24 hours, and the number of colonies was counted. The figures in the tables give the average number of colonies per cubic centimeter of broth.

The results are shown in Tables IV, V, and VI. These tables show clearly that beef heart and rabbit tissue extracts have a decided influence on bacterial lag, and, moreover, that the effect varies with the amount of extract added. The smaller the amount of extract, the longer is the lag phase; and when the concentration of the accessory substances is too low, the results are the same as in the broth controls.

Although this is not a study of lag, the bearing of these experiments

TABLE VI.
Effect of Rabbit Tissue Extracts on Bacterial Lag.

Test culture, pneumococcus.							
Plating interval.	No. of colonies per cc. of culture at time of plating						Broth C.*
	Stomach extract.		Heart extract.		Muscle extract.		
	0.5 cc.	0.1 cc.	0.5 cc.	0.1 cc.	0.5 cc.	0.1 cc.	
<i>hrs.</i>							
0	15,000	15,000	18,000	17,000	17,000	17,000	18,000
2	22,000	15,000	17,000	17,000	18,000	17,000	18,000
4	38,000	26,000	60,000	25,000	36,000	9,500	14,000
5	71,000	53,000	73,000	46,000	71,000	7,000	5,000
6	180,000	90,000	148,000	87,000	210,000	4,500	7,000
24	+++	+++	+++	+++	+++	Sterile.	Sterile.

* Two broth controls were run with the same results.

on the explanation of this phenomenon should be noted. The fact that even under favorable conditions there is a quiescent period before active growth commences, confirms Chesney's assumption of recuperative adaptation. It appears, however, that recuperation is not possible unless some substances are present which favor the active initiation of growth. Ordinarily this is apparently supplied by the disintegrating cells. This interpretation would account for the need of heavy inocula for successful transplants of cultures.

Besides throwing some light on the lag phenomenon, these experiments confirm the fact that the tissue extracts contain some

substances favorable to bacterial growth. All of them in certain concentrations convert an unfavorable substrate where growth does not occur into a favorable one. This effect is produced in the case of rabbit heart and stomach extracts by the addition of only 0.1 cc., while in the case of muscle extract of the same animal a larger amount must be added to obtain that result. The influence of the extract must, therefore, depend on the concentration of some substance or substances in them. Whatever the nature of these substances, it is of interest to note that they are present in lesser concentration in extract of muscle than in that of heart or stomach mucosa.

Effect of Nasal Secretions on Growth of Bacteria.—Shearer has reported experiments showing the stimulating action of nasal secretions on the growth of the meningococcus. It seemed worth while, in view of the results recorded above, to confirm and possibly extend these observations. The tests were limited to nasal washings and saliva.

The first tests were made with nasal secretions. These were mainly confirmatory of the work of Shearer. The secretions were obtained by blowing the nose into strips of sterile gauze. These were put into saline solution or alcohol, and kept for 3 days. The saline extract was divided into two parts, one of which was filtered through a Berkefeld candle, while the other was autoclaved. The alcoholic extract was desiccated to dryness, and then taken up in saline solution.

These extracts were added in 1 cc. and 0.5 cc. amounts to tubes containing 10 cc. of glucose agar and 1 cc. of sheep serum, and plates were poured. The plates were sown with broth suspensions of meningococci; blood and serum plates served as controls. Both saline extracts were superior to the alcoholic extract, though the latter improved the growth as compared with the serum plate. The filtered extract was more effective than the heated one. In no case was the growth so good as on the blood agar plate. The results showed clearly that the nasal mucus contains some substance which favors growth.

The subsequent experiments were modified with a view to determining whether pathogenic bacteria were capable of growing in the

secretions, and, if so, whether there was any difference in their ability to do so. For this purpose saline washings of the nose of apparently normal individuals as well as saliva were used; 50 cc. of warm saline solution were used for each washing. These were collected in sterile bottles, filtered through a Berkefeld candle, tested for sterility, and tubed in 2 cc. amounts in small test-tubes. The saliva was collected in the course of the day, filtered through a Berkefeld candle, and tubed in the same way.

Growth in Nasal Washings.—The nasal washings of five adult individuals and one rabbit were used. The washings were all ob-

TABLE VII.
Growth of Pathogenic Bacteria in Nasal Washings.

Source of washings.	Culture.															
	Meningococcus.				Gonococcus.		Strepto- coccus.		Pneumo- coccus.		<i>B. diph- theria</i> .		<i>B. per- tussis</i> .		<i>B. influ- enza</i> .	
	Para.		Normal.													
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.
Rabbit.	—	—	—	—	—	—	+	+	+	+	—	—	+	+	—	—
W.	+	+	+	+	—	±	+	+	+	+	—	—	+	+	+	+
D.	—	—	—	—	—	Tr.	+	+	+	+	—	—				
Z.	+	+	—	—	—	Contaminated.				+	+	±	±		+	+
K.	+	+	+	+	—	“	+	+	+	+	—	—	+	+	+	+
T.	—	—	—	—	—	±	+	+	+	+	+	+	+	+	—	—
Saliva K.	—	—	—	—	—	—	—	—	—	—	±	+	—	—	—	—
“ Z.	—	—	—	—	—	—	—	—	—	—	±	+	—	—	—	—

tained on the same day and treated in the same manner. The inoculations were made by diluting 0.1 cc. of a 6 hour broth culture in 5 cc. of broth and adding 0.1 cc. of the suspension to each tube. Growth was determined by turbidity and checked by means of Gram-stained films. The results are shown in Table VII.

The washings were made as nearly as possible in the same manner. The rest of the procedure was identical. The same cultures were used for inoculating all the tubes. Yet there were marked individual and specific differences. A given organism grew in the washings of one individual and not in those of another. Chemical tests revealed no differences in the washings. They were all slightly acid

when fresh, but reverted to alkalinity on standing (probably due to a loss of carbon dioxide). No amino nitrogen and only a trace of nitrogen could be detected in 5 cc. of the washings. Reduction tests for glucose were also negative. Nevertheless they offered a favorable substrate for growth while saline controls were negative. No growth appeared in the saliva tubes. Evidently the nasal secretions of some individuals contain substances which actively stimulate growth, while saliva does not.

Relative Effect of Saline and Ether Extracts.—To ascertain the relative importance of the water-soluble and fat-soluble fractions, com-

TABLE VIII.
Growth Accessories in Ether and Saline Extracts.

Extract.	Culture.					
	Streptococcus.	Pneumococcus.	<i>B. diphtheriae</i> .	Meningococcus. Normal.	<i>B. pertussis</i> .	<i>B. influenzae</i> .
Saline.....	+	+	++	+	±	—
Ether.....	±	—	±	±	—	—
Mixture.....	+	+	+	+	±	—
Peptone.....	—	—	±	—	—	—

parative tests were made with saline and ether extracts. Blood clot was used for this experiment; it is rich in vitamins and has a markedly stimulating effect on the growth of bacteria. Equal portions of blood clot, from which the serum had been completely removed by centrifugalization, were weighed out. One portion was suspended in ten volumes of saline solution, the other in ten volumes of alcohol ether (1:3). The alcohol was added first to desiccate the clot and the ether was then added. The saline extract was filtered through a Berkefeld candle. The ether extract was decanted, evaporated nearly to dryness under suction, and the residue taken up in an amount of saline solution equal to the original volume.

Media containing these extracts were then prepared. This time a peptone phosphate solution was used as a base in place of nutrient broth to eliminate all traces of vitamins from other sources. 1 cc. of the respective extracts, as well as a mixture of the two, was added to tubes containing 5 cc. of the peptone solution. These were inoculated in the usual manner and incubated at 37°C.

The results are tabulated in Table VIII. It is evident that the saline extract contains some substances which favor the growth of bacteria, while the ether extract is practically devoid of them. Moreover, inoculations of the various organisms made into the extracts directly failed to give growth. Neither the extract alone nor the peptone alone was capable of supporting growth. The two together constituted a favorable medium. Similar results were obtained with saline and ether extracts of beef heart.

Nature of the Growth Accessory Substances in These Extracts.—The question naturally arises: To what is the favorable effect of these extracts due? Is the improvement of the medium attributable to the addition of food accessory substances or merely to an enrichment in its nutritive quality? This question is naturally difficult to answer, but some experiments have been made which indicate that we are dealing with a vitamine stimulation.

1. If the effect of the extracts is the result of a high content of nutritive substances, bacteria should grow abundantly in them. Tests showed that although moderate growth occurred in the concentrated extracts, none appeared when the extract was diluted five times with saline solution.

2. It has been assumed that the addition of serum enhances the value of the medium, because of the "native" protein. The proteins of the extract were precipitated with alcohol and removed by centrifugalization. The supernatant fluid freed from the alcohol by evaporation under vacuum had the same effect as the original extract.

3. There is some contradiction in the literature regarding the heat stability of vitamins, but it is generally agreed that high temperatures partially or completely destroy these substances (Chick and Hume). On the other hand, amino-acids or carbohydrates, the essential nutritive substances for bacteria, are not affected by heat.

Extracts were prepared in different ways, by employing heat, and the effect on growth was compared with those prepared at the ice box temperature. Lean fresh beef heart was used. Equal weights were macerated and taken up with nine volumes of saline solution. These suspensions were treated respectively as follows: (1) kept in ice box over night and steamed in an Arnold sterilizer for 1 hour;

(2) kept in ice box over night and filtered through a Berkefeld candle; (3) kept at 55°C. over night and heated in an Arnold sterilizer for 1 hour; (4) kept at 55°C. over night and filtered through a Berkefeld candle; (5) extracted by boiling for 1 hour and sterilized in an Arnold sterilizer for 1 hour; (6) extracted by boiling for 1 hour.

The relative effect of these six extracts was tested in the usual way. The results with the agar slants were more striking than those obtained with the broth and are shown in Table IX.

TABLE IX.

Effect of Heat on the Concentration of Substances Favoring the Growth of Bacteria.

Extract.	Agar slants. Relative abundance of growth after 24 hrs. at 37°C.					
	Culture.					
	Strepto- coccus.	Pneumo- coccus.	<i>B. diphtheriæ.</i>	Meningococcus.		<i>B. pertus- sis.</i>
				Normal.	Para.	
Ice-Arnold.....	++	++	—	±	—	±
Ice-filter	++++	++++	++	+++	++	+
55°C.-Arnold	+	+	—	±	—	±
55°C.-filter.....	++++	++	++	+++	++	+
Boiling-Arnold.....	+	+	—	±	—	±
Boiling	++	+	±	±	—	±
Plain agar	+	+	1 colony.	±	—	—

The results shown in Table IX indicate clearly that heat destroys to some extent the substances which favor bacterial growth. Extracts prepared at ice box temperature or at 55°C. are superior to those obtained by heating, and the longer the heating, the less effective are the extracts. The fact that these substances are heat-labile supports the view that they belong to the class of growth accessory substances.

SUMMARY.

The growth of all the pathogenic bacteria studied was favorably influenced by the addition of small amounts of tissue extracts.

Beef heart, rabbit and cat tissues, and human nasal secretions contain substances favorable to the growth of the organisms tested. The mucosa of different organs, spleen, liver, and kidney, are relatively rich in these substances, while muscle is relatively poor. The favor-

able effect of the extracts is manifested by an enhancement of growth and a reduction of lag.

The water-soluble substances are apparently the ones essential for bacterial development; the ether extract has no effect on growth.

Experiments are reported which indicate that the substances in question belong to the class of so called vitamins.

BIBLIOGRAPHY.

- Agulhon, H., and Legroux, R., Contribution à l'étude des vitamines utilisables à la culture des microorganismes. Application au bacille de l'influenza (*B. de Pfeiffer*), *Compt. rend. Acad.*, 1918, clxvii, 597.
- Bottomley, W. B., Some accessory factors in plant growth and nutrition, *Proc. Roy. Soc. London, Series B*, 1914-15, lxxxviii, 237.
- Chesney, A. M., The latent period in the growth of bacteria, *J. Exp. Med.*, 1916, xxiv, 387.
- Chick, H., and Hume, E. M., The effect of exposure to temperatures at or above 100°C. upon the substance (vitamine) whose deficiency in a diet causes polyneuritis in birds and beri-beri in man, *Proc. Roy. Soc. London, Series B*, 1917-19, xc, 60.
- Cole, S. W., and Lloyd, D. J., The preparation of solid and liquid media for the cultivation of the gonococcus, *J. Path. and Bacteriol.*, 1916-17, xxi, 267.
- Gordon, M. H., Hine, T. G. M., and Flack, M., An experimental study of the cultural requirements of the meningococcus, *Brit. Med. J.*, 1916, ii, 678.
- Lloyd, D. J., On vitamines, amino-acids, and other chemical factors involved in the growth of the meningococcus, *J. Path. and Bacteriol.*, 1916-17, xxi, 113.
- Loeb, J., and Northrop, J. H., Nutrition and evolution. Second note, *J. Biol. Chem.*, 1916, xxvii, 309.
- McCollum, E. V., and Davis, M., The essential factors in the diet during growth, *J. Biol. Chem.*, 1915, xxiii, 231.
- McCollum, E. V., and Simmonds, M., The nursing mother as a factor of safety in the nutrition of the young, *Am. J. Physiol.*, 1918, xlvi, 275.
- Mockenridge, F. A., Some effects of growth-promoting substances (auximones) on the soil organisms concerned in the nitrogen cycle, *Proc. Roy. Soc. London, Series B*, 1917, lxxxix, 508.
- Müller, M., cited by Chesney.
- Northrop, J. H., The rôle of yeast in the nutrition of an insect (*Drosophila*), *J. Biol. Chem.*, 1917, xxx, 181.
- Osborne, T. B., and Mendel, L. B., The rôle of vitamines in the diet, *J. Biol. Chem.*, 1917, xxxi, 149.
- Pacini, A. J. P., and Russell, D. W., The presence of a growth-producing substance in cultures of typhoid bacilli, *J. Biol. Chem.*, 1918, xxxiv, 43.
- Penfold, W. J., cited by Chesney.
- Shearer, C., On the presence of an accessory food factor in the nasal secretion, *Lancet*, 1917, i, 59.

STUDIES ON ORGAN TRANSPLANTATION.

I. TRANSPLANTATION OF THE THYROID GLAND WITH INTACT BLOOD SUPPLY.

By K. KAWAMURA, M.D.

(From the Division of Experimental Surgery of the Mayo Clinic, Rochester, Minn.)

PLATES 4 TO 6.

(Received for publication, April 4, 1919.)

There are two methods of free grafting of a gland: the transplantation of pieces of tissue, and the transplantation of the whole organ with anastomosis of its blood vessels to those of the same person or of another person. In 1892 von Eiselsberg transplanted the thyroid gland in a cat. He removed half of the gland and transplanted it into the abdominal wall. Several weeks later he extirpated the other half of the gland. The animal remained in good condition but died when the transplanted thyroid was removed. Since that time many instances of thyroid grafting, both experimental and clinical, have been reported, and it has been shown that thyroid grafts in animals take and functionate.

Fairly effective results were obtained by Cristiani (1904, 1906) after transplantation of thyroid tissue in the subcutaneous cellular tissue, by Payr (1906) after transplantation in the spleen, and by Kocher after transplantation in the bone marrow. On the other hand, there are also many reports of negative results. Enderlen considered the functional activity of transplanted thyroid limited and temporary, as a consequence of his experiments in which cats and dogs died even after a period of 6 months. Bircher, Leichner and Köhler, Stieda, and others implanted thyroid gland into the subcutaneous tissue or bone marrow of patients, with no permanent improvement. In 1914 von Eiselsberg reported the transplantation of thyroid and parathyroid in man. It seemed to him at first that the grafting was effective, but all efforts finally proved unavailing. He believes that the function of the transplanted pieces of glands is probably only temporary.

The development of vascular surgery has made it possible to transplant whole organs with their related vessels. In 1905 Carrel and Guthrie extirpated the thyroid gland of a dog, placed it in isotonic salt solution for a few minutes,

and replaced it into the neck, anastomosing the blood vessels but with reversal of the circulation. The arterial blood entered the gland through the thyroid vein and the venous blood flowed from the gland to the jugular vein through the thyroid artery. 11 days after the operation an exploratory opening of the neck was made. The color and consistency of the gland were normal. 8 months after the operation the gland was normal in size and consistency. Later, Carrel (1909) extirpated a large parenchymatous goiter in a dog and replanted it. He then cut transversely the common carotid and united its central end to the peripheral end of the internal jugular. The central end of the jugular was sutured to the peripheral end of the carotid. The size of the gland increased enormously. At the same time, clear fluid exuded from the surface of the gland but diminished and stopped completely after more than a week.

Stich and Makkas performed a series of thyroid transplantations. After the dissection of the upper thyroid artery to the point of its departure from the common carotid, they resected a rhomboid flap of the carotid in connection with the thyroid artery and inserted it in the corresponding hole of the other carotid. The internal jugular vein was cut transversely just beneath its union with the lower thyroid vein, and sutured with the external jugular vein by end to side or end to end anastomosis. At the same time the other half of the gland was extirpated to obtain, immediately, sufficient function for the transplanted thyroid. Of three autoplasmic transplantations, two were positive; that is, 51 and 245 days respectively after the operation the circulation was intact and the gland, both macroscopically and microscopically, was normal.

The gland was transplanted seven times homoplastically, but no positive result was obtained. An obstruction was almost always found at the point of the venous anastomosis, which brought about necrosis and reabsorption of the gland.

Borst and Enderlen similarly transplanted the thyroid of dogs and goats. Their method differed somewhat from the procedure employed by Stich and Makkas in that they used, for the grafting of the thyroid artery, the complete segment of the carotid with the thyroid artery. In seven instances they extirpated the thyroid and replanted it in the same animal. In two of the seven animals (20 and 122 days after operation) the glands were in good condition. In the other animals necrosis, hemorrhagic infarct, degenerative metamorphosis, etc., took place, due to the thrombosis of the thyroid vein. They attempted homoplastic transplantation seven times (in dogs and goats), but none was successful. They also attempted in three instances to transplant the thyroid from man to man by the employment of blood vessel sutures. The upper pole of the gland was transplanted into the axilla or the elbow. The results in every case were fruitless.

Goodman also investigated this problem. He made a biterminal suture of a segment of the attached carotid of the severed vessel of the host, and an end to end suture of the thyroid vein with the central end of the external jugular of the opposite side. He performed three autoplasmic and twenty-seven homoplastic transplantations, and observed them from 24 hours to 112 days after the operation. In autoplasmic transplantation he succeeded in two instances (the dura-

tion of life was 4 and 23 days respectively) in retaining the thyroid gland in its normal state; while in homoplastic transplantation the gland remained intact for a short time only, and then showed evidence of absorption.

The results of the investigations with regard to thyroid transplantation as reviewed in the literature did not seem uniform and we were therefore induced to investigate the problem further. We chose for our experiments the transplantation of the gland through blood vessel sutures because of the favorable restoration of the circulation of the gland by this method.

EXPERIMENTAL.

All the experiments were performed on dogs. Since the relation of the thyroid to its vessels in the dog is different from that in man, before describing the technique it may be well briefly to note this difference. In a dog the glands are spindle-shaped, situated beside and beneath the larynx, usually separated from each other, and more or less covered with muscle. The dog, especially the bulldog, has a relatively large thyroid, which is not always proportional to the size of the animal; a small dog may have remarkably large glands due to the development of the so called colloid goiter. The arterial blood reaches the gland mainly through the superior thyroid artery, which arises from the common carotid and enters the gland at its upper pole after having made an upward convex curve. Very rarely this artery enters the gland near its middle and ramifies with many branches. The inferior thyroid artery is very small; accordingly it may usually be ignored in the transplantation of the thyroid. The venous blood flows out through two veins, the superior and inferior thyroid veins. The superior thyroid vein leaves the upper pole of the gland and empties into the internal jugular vein, while the inferior thyroid vein, consisting of two stems, opens into the same vein further down. The diameter of the superior thyroid artery and thyroid veins is scarcely greater than 1.5 to 2.5 mm. It is, therefore, almost impossible to anastomose these vessels.

The operation was performed under intratracheal ether anesthesia. The Carrel (1907) technique of blood vessel suturing was employed, and special attention was paid to rigid asepsis. A longitudinal median

incision was made in the neck and the superficial muscles were separated. The thyroid gland was dissected from the surrounding tissue, the superior thyroid artery and superior and inferior thyroid veins being left intact. In most instances the gland was extirpated with a portion of the internal jugular vein and a segment of the common carotid artery, connecting the thyroid veins and the superior thyroid artery respectively. The gland was immediately wrapped in a sponge saturated with warm salt solution. After a few minutes the gland was transplanted to the other side of the neck of the same dog, or into the neck of another dog, where the thyroid had been previously removed. The segment of common carotid was inserted in the place of the other below the point of outlet of the thyroid artery. The peripheral end of the internal jugular vein was united to the central end of the internal or external jugular vein of the recipient by end to end anastomosis. In one case the gland with a rhomboid flap of the carotid at the point of outlet of the superior thyroid artery was removed (Stich's so called patching method) and replaced in the wound in the neck. In two animals the peripheral end of the internal jugular vein was united to the wall of the external jugular vein (end to side). Moreover, in two cases, after resection of half of the spleen, the thyroid was transplanted to the splenic vessels of the same animal. In these instances the superior thyroid artery was sutured to the splenic artery, and the internal jugular vein to the splenic vein by end to end anastomosis. The time required for the extirpation of the gland and its complete transplantation was usually from 1 to 2 hours. The clamp on the vein was removed first and then that on the artery. As soon as the clamps were unfastened the gland became normal in color; it was somewhat distended. Several days after the operation the wounds were opened and the condition of the transplanted thyroid gland was examined. As a rule, if the gland appeared normal the other intact thyroid was fixed in 10 per cent formaldehyde for microscopic examination.

Both autotransplants and homotransplants of the gland were made, as shown in Table I. A total of eight autoplasic and seven homoplasic transplantations was made. Two autoplasic transplants (Experiments 2 and 3) and one homoplasic (Experiment 9) are described below.

Experiment 2 (Dog 2).—Adult bulldog, male; weight 12 kilos.

Sept. 18, 1918. Placed under ether anesthesia and the right thyroid gland transplanted to the left side of the neck. The segment of the right carotid artery, from which the superior thyroid artery arose, was implanted by end to end anastomosis into the left carotid. The peripheral end of the right internal jugular vein was anastomosed to the cardinal end of the left external jugular vein. The excised gland had been left in a salt pack for a short time. After the removal of the clamps the pulsation of the superior thyroid artery was very evident and the circulation through the gland was reestablished so that it immediately became normal in color. The extirpation of the gland and its complete transplantation occupied $1\frac{1}{2}$ hours. The wound was closed according to the routine technique of the laboratory.

Sept. 20. The animal was in good condition and had a normal appetite.

Oct. 9. Second operation. The weight of the animal at this time was 13.1 kilos. The transplanted thyroid appeared perfectly normal. The intact left thyroid was removed. The animal recovered from the operation and never showed any signs of tetany.

Oct. 29. The animal died early in the morning (41 days after the first operation and 20 days after the second).

Autopsy.—Performed shortly after death. The wound had healed completely; the cause of death could not be found. The transplanted thyroid was normal in size, color, and consistency and appeared normal on section. The capsule of the gland was not markedly thickened. The implanted blood vessels were patent. The lines of union were covered with epithelium and so smooth that they were found with difficulty.

Microscopic Examination.—The transplanted thyroid showed no difference from the control gland. The capsule of the gland was not increased in thickness. The follicles were normal in size and about the same in both the central and peripheral parts of the gland; they were lined with cuboidal epithelium, and filled with a normal amount of colloid. The staining reaction was normal; mitoses were not present. The interstitial connective tissues were not increased as compared with the control specimen. Increase of blood vessels was not visible. No leucocytic infiltration could be found (Figs. 1 and 2).

Experiment 3 (Dog 3).—Adult collie, female; weight 21.3 kilos.

Sept. 19, 1918. Etherized and the left thyroid gland transplanted to the right side of the neck. The gland was small, as was also the internal jugular vein. The left thyroid was displaced to the right with a segment of the common carotid and the internal jugular vein. The segment of the left carotid was inserted into the right carotid. The peripheral end of the left internal jugular vein was implanted into the wall of the right external jugular by a terminolateral anastomosis. The operation was difficult owing to the different sized vessels. Nevertheless, it was, on the whole, satisfactory. There was no leakage at the line of suture. The circulation was reestablished about $1\frac{1}{2}$ hours after the extirpation of the gland. The wound was closed in the routine manner.

TABLE I.
Summary of Experiments.

Experiment No.	Operation.	Duration of observation.	Results.			Remarks.
			Condition of blood vessels.	Condition of transplanted thyroid.	Microscopic examination.	
Autoplastic transplantation.						
1 (Dog 1).	1918 Sept. 11. Transplantation of right thyroid with a segment of common carotid to left side. Oct. 30. Exploratory opening of neck.	49 days	Left common carotid pulsating everywhere; superior thyroid artery and inferior thyroid vein thrombosed.	Entirely absorbed.		At first operation great hemorrhage from peripheral end of right common carotid due to slipping ligature. Lower part of wound slightly infected. Dog still living. Oct. 29. Died.
	2 (" 2).	Sept. 18. Transplantation of right thyroid with a segment of common carotid to left side. Oct. 9. Extirpation of the original left thyroid.	61	Arterial and venous lumen patent. Line of union covered with endothelium, and smooth. No thrombosis or clots.	Size, color, and consistency absolutely normal.	No necrosis. Appeared normal.
3 (" 3).	Sept. 19. Transplantation of left thyroid with a segment of common carotid to right side. Termination lateral anastomosis between left internal and right external jugular vein.	62	Arterial and venous lumen patent. Line of union smooth. No thrombosis or clots.	Appeared entirely normal.	No necrosis. Appeared almost normal.	Nov. 30. Died.

4 (Dog 4).	Sept. 21. Transplantation of left thyroid with a segment of common carotid to right side. Terminolateral anastomosis between left internal and right external jugular vein.	4	Arterial anastomosis in good condition. Very small clot at mouth of external jugular vein.	Almost same size as the other. Fairly dark and hard but mostly translucent.	Almost normal with exception of a few congested blood vessels.	Both thyroids small. Congestion of venous blood at the point of anastomosis due to a bend of transplanted internal jugular vein which was too long. Animal died of dis-temper.
5 (" 5).	Sept. 23. Replacement of left thyroid. Rhomboid flap was taken from left common carotid. Oct. 9. Exploratory opening of the neck.	18	Arterial suture intact, but vein stopped up.	Normal size; soft. Surface of section pale, not translucent.	Follicle and colloid entirely disappeared. Abundant leucocytic infiltration and proliferation of connective tissue.	Both thyroids very small. Oct. 11. Died.
6 (" 6).	Nov. 29. Replacement of right thyroid with a segment of right common carotid Dec. 16. Resection of half of spleen. Transplantation of left thyroid into splenic vessels. Jan. 13, 1919. Exploratory laparotomy.	1	All sutures in good condition.	Appears normal.	Almost normal; a few leucocytic infiltrations.	Nov. 30. Died. Cause of death unknown.
7 (" 7).		28	Obstruction at point of arterial anastomosis.	Normal size but dark in color. Whole gland necrotic.	Necrosis.	Superior thyroid artery entered at the middle part of the gland.

TABLE I—*Concluded.*

Experiment No.	Operation.	Duration of observation.	Results.			Remarks.
			Condition of blood vessels.	Condition of transplanted thyroid.	Microscopic examination.	
8 (Dog 8).	1918 Dec. 23. Transplantation of right thyroid into splenic vessels.	days 21	Obstruction at point of arterial anastomosis; thrombosis in vein.	Gland diminished to size of tip of thumb. Surface of section anemic; no blood came out.	Necrosis.	At the first operation spleen not resected.
	Jan. 13, 1919. Exploratory laparotomy.					
Homoplastic transplantation.						
9 (Dog 9).	Sept. 25. Transplantation of left thyroid with a segment of common carotid of left neck. Internal jugular vein united to external jugular.	6	Line of suture in good condition. Caliber of artery and vein patent.	Size, color, and consistency almost normal. Longitudinal section showed a thin brownish and somewhat gelatinous part along the posterior border.	Almost normal. Septum between follicles increased more or less in thickness. Follicles filled with colloid. Deposit of lime and loosed epithelium visible here and there in follicles.	Both glands very large. Oct. 1. Animal died of distemper.
					Interstitial tissues increased. Follicles filled with leucocytes, detritus, and fibrin. Here and there deposit of lime.	
10 (" 10).	Sept. 26. Transplantation of left thyroid with a segment of common carotid to right neck. Ends of internal jugular vein united to each other by end to end anastomosis.	18	Right common carotid pulsating everywhere.	Gland kept its original size; color pale red. No hemorrhage on incision.		
	Oct. 14. Exploratory opening and proof excision of the gland.					

11 (Dog 11).	Sept. 27. Transplantation of right thyroid with a segment of common carotid to left neck. Oct. 14. Exploratory opening. Extirpation of right intact thyroid.	24	Thrombosis in common carotid. Obstruction of internal jugular vein along the suture line.	Gland kept almost original size; softer than normal. Cut surface brown, dim, not translucent.	Capsular thickening. No trace of follicles or colloids.	At second operation the veins on anterior portion of gland were visible and filled with blood. Oct. 21. Animal died with typical symptoms of tetany. A week after first operation large hematoma formed in the neck. Oct. 5. Died.
12 (" 12).	Sept. 28. Transplantation of left thyroid with a segment of common carotid to left neck.	63	Transplanted common carotid and internal jugular vein obstructed.	Entirely disappeared.		
13 (" 13).	Sept. 30. Transplantation of left thyroid with a segment of common carotid to left neck.	5	Thrombosis in common carotid and inferior thyroid vein.	Enlarged; cut surface dark red.	Septum increased in thickness. Follicles filled with detritus and fibrin. Nuclear staining weak.	
14 (" 14).	Oct. 1. Transplantation of right thyroid with a segment of common carotid to left neck. Oct. 30. Exploratory opening.	29	Thrombosis in common carotid.	Atrophy of gland to size of tip of little finger.		Caliber of internal jugular vein very small.
15 (" 15).	Oct. 4. Transplantation of right thyroid with a segment of common carotid to left neck. Oct. 30. Extirpation of right intact thyroid.	27	Thrombosis in common carotid, in inferior thyroid vein, and in internal jugular vein.	Diminished to size of tip of thumb. Consistency soft.	Follicles and colloid entirely disappeared. Here and there clumps of lymphocytes. Abundant proliferation of connective tissue.	Animal died with symptoms of tetany day after second operation.

Oct. 9. Second operation. At this time the animal weighed 21.4 kilos. The transplanted thyroid gland appeared normal. The intact right thyroid was removed and fixed in formaldehyde.

Nov. 20. The animal died during the preceding night (62 days after the first operation and 42 days after the second).

Autopsy.—Performed shortly after death. The wound had healed by first intention; there were slight adhesions at the site of operation. The transplanted thyroid appeared entirely normal. The blood vessels were patent, and the site of anastomosis was in excellent condition. The cause of death could not be determined.

Microscopic Examination.—The transplanted thyroid was practically normal. The thickness of the capsule of the gland was normal. No difference was seen in the size and form of the follicles, in the colloid content, or in the quantity of interstitial connective tissue, in comparison with the other gland of the same dog. Cuboidal epithelium lined the follicles as in the control section. The gland contained a large number of blood vessels. Here and there clumps of a few leucocytes were visible (Figs. 3 and 4).

Experiment 9 (Dog 9).—Adult mongrel, male; weight 5 kilos.

Sept. 25, 1918. The left thyroid of another dog was transplanted, under ether anesthesia, to the left side of the neck by the insertion of a segment of the common carotid into the carotid. After the dissection and removal of the left thyroid gland, about 3 cm. of the left common carotid were resected. The peripheral end of the internal jugular vein of the donor was united to the central end of the external jugular of the recipient. The transplanted thyroid was very large, as was also the internal jugular vein. The operation was easily performed. After 1½ hours the circulation through the gland was reestablished satisfactorily. Closure of the wound was made as usual. The animal recovered from the operation but developed distemper.

Oct. 1. The animal died during the night.

Autopsy.—Death due to distemper. Both the arterial and the venous anastomoses were in good condition; the caliber of the vessels was well preserved. The transplanted thyroid appeared normal in size, color, and consistency. On section it was normal in appearance except in the posterior border where the tissue was somewhat brown in color and was more gelatinous than the other part.

Microscopic Examination.—The capsule of the gland was more or less thickened. The interlobular septum also appeared to be thickened. The size of follicles and their colloid content were as well preserved as in the autografts. The staining reaction appeared normal. Few follicles contained desquamated cells in their lumen. Some of these cells were calcified. There was a small amount of leucocytic infiltration. The vascularization of the gland was normal (Fig. 5).

Eight autoplasmic and seven homoplasmic transplantations of the thyroid gland were performed. In two of the eight autoplasmic transplantations the gland was transplanted to the splenic vessels, in six

to the neck. The first two cases were examined by exploratory laparotomy 21 and 28 days respectively after the first operation. It was found that the glands had become necrotic because of thrombosis. It seems almost impossible to get successful results by anastomosing such a small vessel as the superior thyroid artery to the splenic artery. Two of the six transplantations to the neck were replaced in the same wounds, and four were made to the other side of the neck. One of these was examined 18 days after the operation and obstruction of the transplanted vein and necrosis of the gland were found. One transplant was examined 49 days after the operation. The gland had entirely disappeared, and the superior thyroid artery and the inferior thyroid vein could not be found, though the pulsation of the common carotid was very evident throughout. The cause of the failure was due to infection of the wound. In four instances (1, 4, 41, and 62 days after the operation), all blood vessels were patent, and the gland appeared, both macroscopically and microscopically, to have preserved its normal structure.

Seven of the homoplastic transplantations were examined in periods of 5, 6, 18, 24, 27, 29, and 63 days after the first operation. In two the segments of the carotid remained free from thrombosis and were covered smoothly with endothelium, although in five instances the transplanted vessels, in whole or in part, were occluded. The thyroid gland showed evidences of remarkable change in proportion to the time elapsed. 6 days after the operation the gland retained its original size and, for the most part, was translucent, but in a part of the posterior border the tissue was beginning to soften. Microscopic examination showed that the size of follicles and colloid content were as well preserved as in the autograft. In the gland which was examined 18 days after the transplantation, the consistency was markedly soft, the color of the section was pale red, and no hemorrhage had occurred at that time, notwithstanding the fact that the gland kept its original size and the carotid was still patent. Microscopic examination showed great degeneration of the gland. After 24 days the microscopic change of the gland increased, and it became gradually smaller. In one case, 63 days after the operation, no trace of the gland was to be found. In brief, our attempts at homoplastic transplantation were unsuccessful.

DISCUSSION.

Our results in thyroid transplantation by blood vessel sutures agree on the whole with the experiences of Stich and Makkas, Borst and Enderlen, and Goodman. In the autoplasic transplantations we succeeded, as they did, in maintaining the glands for a long time in their original shape and structure, and apparently in function. In the series of homoplasic transplantations, on the contrary, the transplant remained intact for a time, then underwent degenerative changes, and, consequently, absorption.

Hesselberg has published a paper concerning a comparison of autoplasic and homoplasic transplantation of thyroid tissue in guinea pigs. She transplanted pieces of glands into the subcutaneous tissue of the abdominal wall and examined them at intervals of from 24 hours to 52 days. In the first stage, comprising the first 4 to 5 days, there was no noticeable difference between the autografts and the homografts. Large parts of both grafts became necrotic in the center, but in the peripheral part only a narrow zone of thyroid tissue was left. In the second stage, extending over the next 7 days, the differences between the two grafts appeared. There was an increase in the number of follicles in the autografts and a corresponding decrease in the size of the necrotic central area. In the homografts a large number of lymphocytes and connective tissue were visible. From the 12th day on, in the third stage, the difference between the grafts was sharply defined. In the autograft the regeneration of the thyroid tissue was steadily progressing and nearly completed after 21 days. In the homograft, on the contrary, the destruction of the follicles progressed with great intensity.

Loeb (1918-19, *a, b*) recently designated the transplantation into nearly related animals and to the nearly related of the same species as "syngenesioplasic" transplantation. He carried out transplantation of thyroid from mother to children, from sister and brother to sister or brother, and in one instance from child to mother in guinea pigs. The results were intermediate between those which Hesselberg obtained after autotransplantation and homotransplantation. In most instances the thyroid behaved, for a time, like an autotransplanted tissue, but gradually an intensive lymphocytic infiltration took place with secondary destruction of the healthy acini. In a smaller number of cases the fibrous tissue also was increased, the fibroblasts behaving like those in cases of homoplasic transplantation.

In one of our cases of homoplasic transplantation also (Experiment 9) the gland showed indications of degeneration on the 6th day after the operation, in spite of the fact that the transplanted blood

vessels were patent. The destruction of the gland increased in the process of time.

Homoplastic transplantation, however, can be successfully made in certain kinds of tissue. At the instant the animal dies the tissues, in general, do not lose their vitality in spite of the arrest of the circulation. On the other hand, individual tissues have a different power in regard to regeneration and repair. Some tissues, such as skin, fascia, periosteum, and bone, which survive for a long time under the interruption of the circulation, can regenerate and repair easily. Such tissues, as is well known, can be transplanted homoplastically with success although the ultimate fate of the transplant is not fully known.

As has been noted, the opinions of authors differ with regard to the fate of the more highly organized tissues, such as the thyroid gland. Cristiani has shown that transplanted and functioning thyroid may be kept in good condition in its new location for a long time, provided the operation is performed with certain precautions. For the successful transplantation, he emphasized that the pieces of gland transplanted must be small and numerous, that thyroid tissue from the same species only will grow, and that living tissue may be transplanted, provided that not more than 10 seconds elapse between the extirpation of the tissue and its transplantation. Cristiani applied also, clinically, the results of his experimental work, and from his experience on patients arrived at the following conclusions. With his method of thyroid grafting it is possible to produce new thyroid organs which not only retain their vitality and functionate, but gradually grow so that small grafts in time become new thyroid glands (*glandulae neothyreoideae*) of different sizes.

Payr (1906) transplanted thyroid gland into the spleen of animals, believing that spleen is suitable soil for healing because of its vascular nature. Of 48 experiments of autoplasmic transplantation, mostly on cats and dogs, he obtained eight promising results. The longest duration of the observation was 271 days. He had likewise made the transplantation of the thyroid gland clinically. A few years ago he reported seven cases of thyroid transplantation for myxedematous patients; they showed marked improvement, but most of them relapsed from 3 to 8 years after the operation. He remarked that organic cerebral defects of patients cannot be improved by thyroid grafting.

Kocher reports several favorable cases of transplantation of pieces of thyroid in patients, using the bone marrow as new soil. He prefers the tibial metaphysis to the spleen on account of the greater ease and safety of the operation and the equally vascular soil; hemostasis is secured by operating in two stages. With regard to the tissue for transplantation pieces from exophthalmic goiter are especially desirable, because the glands are remarkably vascular, with comparatively firm structure, and because their proper parenchyma surpasses considerably the supporting substance. He stated that there are no grounds for the belief, based on animal experiment, that homoplastic transplantation cannot also succeed in man.

There are some reports of conflicting opinions with reference to these favorable results. Bircher implanted pieces of thyroid into the subcutaneous tissue of the occipital region in three cretins. After short improvement there was relapse into the former state. On histologic examination of the grafts he found that no normal thyroid tissue was left, but necrosis and connective tissue had replaced the whole. His observations made him skeptical as to the permanent function of homoplastic implanted thyroid. Leichner and Köhler were of the same opinion as Bircher. Stieda reported three cases in which the grafting was done on myxedematous children who were more or less idiotic. After operation they showed signs of remarkable, rapid improvement, which shortly came to a standstill. Schaack and Müller repeatedly transplanted thyroid tissue into the bone marrow or into the subcutaneous tissue, with no permanent improvement. The thyroid grafts which Worobjew and Perimow made on a myxedematous patient healed, but subsequently became smaller and were totally absorbed. Von Eiselsberg (1914) also, from his experience with patients, expressed doubt that successful results could be obtained.

Thus, while some observers admit the feasibility of thyroid grafting, others deny it. The negative results of our experiments do not warrant denial of the positive results of Cristiani, Payr, Kocher, etc., but, as Bircher has already noted, we should not lightly pass over the possibility that implanted pieces of tissue will have an influence as long as the colloid exists, that its absorption occurs very slowly, and finally, that the moment the colloid is absorbed its influence stops. Furthermore, although many successful cases of piecemeal homoplastic transplantation on patients have been reported, they are not always without objection, because one important factor is wanting in all; namely, the subsequent removal and microscopic examination of the tissue. In short, for permanent good results we must expect to work further in this direction. Several points must be considered in the successful take of transplanted tissue: (1) The avoidance of

injuring the tissue, and a sufficient blood supply. In this connection the transplantation with intact blood supply is the better method. (2) In the organ transplantation, especially by means of blood vessel sutures, aseptic precaution must be observed as rigidly as possible. If not, the occlusion of the transplanted vessels or necrosis of the organ may easily occur. Concerning asepsis in operations on blood vessels Carrel (1907) states: "It seems that the degree of asepsis under which general surgical operations can successfully be performed may be insufficient for good results in vascular operations." In our first case of autoplasmic transplantation the results immediately after the operation were satisfactory, but the lower part of the wound was, unfortunately, slightly infected. At the exploratory opening of the neck, 49 days after the operation, we found that the transplanted common carotid was patent, but that the gland had entirely disappeared, due to infection.

Besides, the rapid work, keeping the gland warm, the avoidance of dryness, rough handling, chemical irritation, close contact with the new soil, and strict arrest of hemorrhage, are all apparently necessary conditions for a good transplantation. If these precautions are used the autoplasmic transplantation is almost always successful. But we failed, as did the others, in the homoplasmic transplantation, notwithstanding the fact that we used similar care. The factors that may have been responsible for the failure are briefly the following.

It is well known that nerves have some control over the nutrition and function of tissues. Raynaud's disease, for example, is supposed to be due to spasm of the arterioles, resulting from peripheral neuritis. If a nerve trunk is injured severely and the peripheral end undergoes degeneration, several phenomena may occur, due to the loss of the trophic function of the nerve, such as dryness of the skin, trophic ulcers, fragility of the nails and hair, arthropathies, etc. It seems to be reasonable, therefore, to suppose that the failure of the graft can be attributed to the section of the nerves which reach the organ.

Crowe and Wislocki in experimental work on suprarenal glands in dogs attributed degenerative changes of the graft to the lack of nerve supply to it. We do not, however, fully concur with their opinion. Salzer and others also emphasize the independence of grafts from the nervous system. Manley and

Marine, in considering the question of whether or not secretory nerves were necessary to the gland, experimented on rabbits. They have demonstrated that such nerve fibers are not essential in order that thyroid tissue may exhibit the characteristic morphologic and physiologic changes known to be associated with great variations in functional activity. Other authors have also found that thyroid tissue may be readily transplanted in widely distant parts of the body. In our experiments on autoplasmic transplantation we obtained good results in spite of complete dissection and removal of thyroids from their surrounding tissues. We cannot therefore consider that the failure of homoplastic transplantation of the thyroid is due to the section of its nerves.

Murphy tried to prove that a homoplastic or heteroplastic graft cannot develop indefinitely on its host. He discovered that the power of the organism to eliminate foreign tissue is due to organs such as the spleen or bone marrow, and that when these organs are less active (by removal of the spleen or the injection of benzol) a foreign tissue (tumor) can develop rapidly after it has been grafted. The grafts became absorbed from 12 to 15 days after the transplantation.

Hesselberg and Loeb attached a peculiar significance to lymphocytic reaction and to proliferation of connective tissue. They believe that the destruction of the thyroid tissue which occurs after the homografting is not caused by a direct primary disintegration or the solution of follicles, due to the action of substances circulating in the body fluids, but depends on the destructive activity of the lymphocytes, and of the connective tissue of the host. The former invade the follicles and destroy them directly; the latter surrounds and compresses, and thus destroys the follicles. Why do the lymphocytes and the connective tissues attack the homografts only, and not the autografts? It is well known that lymphocytes and connective tissue appear usually as the response to the reaction of foreign bodies. They may be commonly discovered around suture materials, such as silk and linen thread, etc., that have been used at operations. It is reasonable, therefore, to assume that homografts which do not take may act as foreign bodies.

The obstacle to success in homoplastic transplantation lies probably in the differences of biochemical factors between the animals. As Ullman expresses it, the cell protoplasm, specific for each organism, varies with the individual; there are as many protoplasms as there are individuals; in homoplastic transplantation the appearance in the body of a foreign protoplasm calls forth ferments into the circulation which destroy the transplanted tissue.

Stich enumerated the following factors as the cause of the destruction of the grafts: primary toxic action of the tissue fluid on transplanted organs; production of an antibody in the organism of the host through foreign albumin; and finally, starvation of the transplanted tissue, owing to the incapacity of the assimilation of materials that are necessary for its growth.

We are still ignorant of the means of eliminating these factors. Lexer (1914) showed that he had succeeded in overcoming some of the biochemical reactions between his animals by prolonged preliminary treatment of the host with tissues

and blood serum taken from the donor. He considered that the possibility of the improvement of homoplastic surgery was most hopeful along this path. Schöne believes that positive results may be obtained in animals through the same nourishment and the same mode of living for a long period of time. Sauerbruch and Heyde demonstrated that direct communication of blood vessels was proved between parabiotic rabbits, and that substances which are soluble in blood, iodine, strychnine, etc., and even bacteria, go from one animal to the other through newly formed vascular channels. The question of whether or not products of metabolism of one animal are pernicious to another has not been solved. Enderlen and his collaborators aimed to make the biochemical differences equal by direct blood vessel anastomosis in dogs, but they could not attain their object. In short, it seems to be most important to discover the method of equalizing as nearly as possible biological differences between donor and recipient.

CONCLUSION.

Our experiments on dogs showed that the thyroid gland which was autoplastically transplanted, by means of various methods of blood vessel anastomosis, could live in good condition and functionate favorably several months after the operation, even after the interruption of the circulation for $1\frac{1}{2}$ hours. They further showed that the circulation through the transplanted blood vessels as well as glands was as good as normal, and that permanent successful results of the homoplastic transplantation of the gland are as yet not possible.

BIBLIOGRAPHY.

- Bircher, E., Zur Implantation von Schilddrüsengewebe bei Kretinen, *Deutsch. Z. Chir.*, 1909, xcvi, 75.
- Borst and Enderlen, Ueber Transplantation von Gefässen und ganzen Organen, *Deutsch. Z. Chir.*, 1909, xcix, 54.
- Carrel, A., The surgery of blood vessels, etc., *Bull. Johns Hopkins Hosp.*, 1907, xviii, 18.
- Carrel, A., Results of the transplantation of blood vessels, organs and limbs, *J. Am. Med. Assn.*, 1908, li, 1662.
- Carrel, A., Thyroid gland and vascular surgery, *Surg., Gynec. and Obst.*, 1909, viii, 606.
- Carrel, A., The transplantation of organs, *New York Med. J.*, 1914, xcix, 839.
- Carrel, A., and Guthrie, C. C., Extirpation et replantation de la glande thyroïde avec reversion de la circulation, *Compt. rend. Soc. biol.*, 1905, lix, 413.
- Cristiani, H., La greffe thyroïdienne chez l'homme, *Semaine méd.*, 1904, xxiv, 81.

- Cristiani, H., and Kummer, E., Ueber funktionelle Hypertrophie überpflanzter Schilddrüsenstückchen beim Menschen, *Münch. med. Woch.*, 1906, liii, 2377.
- Crowe, S. J., and Wislocki, G. B., Experimental observations on the suprarenal glands with especial reference to the functions of their interrenal portions, *Bull. Johns Hopkins Hosp.*, 1914, xxv, 287.
- von Eiselsberg, A., Ueber erfolgreiche Einheilung der Katzenschilddrüse in die Bauchdecke und Auftreten von Tetanie nach deren Exstirpation, *Wien. klin. Woch.*, 1892, v, 81.
- von Eiselsberg, A., Zur Frage der dauernden Einheilung verpflanzter Schilddrüsen und Nebenschilddrüsen, Zugleich ein Beitrag zur post-operativen Tetania parathyreopriva, *Arch. klin. Chir.*, 1914, cvi, 1.
- Enderlen, Untersuchungen über die Transplantation der Schilddrüse in die Bauchhöhle von Katzen und Hunden, *Mitt. Grenzgeb. Med. u. Chir.*, 1898, iii, 474.
- Enderlen, Hotz, and Flörcken, Ueber Parabioseversuche durch direkte Gefäßvereinigung, *Beitr. klin. Chir.*, 1910, lxx, 1.
- Goodman, C., The transplantation of the thyroid gland in dogs, *Am. J. Med. Sc.*, 1916, clii, 348.
- Hess, J. H., and Strauss, A. A., Autotransplantation and homotransplantation of the thyroid gland using the thyroid capsule as the seat of transplantation, *Arch. Int. Med.*, 1917, xix, 518.
- Hesselberg, C., A comparison of autoplasmic and homeoplasmic transplantation of thyroid tissue in the guinea pig, *J. Exp. Med.*, 1915, xxi, 164.
- Hesselberg, C., and Loeb, L., Successive transplantation of thyroid tissue into the same host, *J. Med. Research*, 1918, xxxviii, 33.
- Kocher, T., Ueber die Bedingungen erfolgreicher Schilddrüsen-transplantation beim Menschen, *Arch. klin. Chir.*, 1914, cv, 832.
- Lechner, H., and Köhler, R., Ueber homoioplastische Epithelkörperchen- und Schilddrüsenverpflanzung, *Arch. klin. Chir.*, 1910-11, xciv, 169.
- Lexer, E., Ueber freie Transplantationen, *Arch. klin. Chir.*, 1911, xcv, 827.
- Lexer, E., Die freie Transplantation, International Society of Surgery, 4th Congress, New York, April 15, 1914; Free transplantation, *Ann. Surg.*, 1914, lx, 166.
- Loeb, L., Syngenesioplasmic transplantation of the thyroid in the guinea-pig, *J. Med. Research*, 1918-19, a, xxxix, 39.
- Loeb, L., Multiple transplantations of the thyroid and the lymphocytic reaction, *J. Med. Research*, 1918-19, b, xxxix, 71.
- Manley, O. T., and Marine, D., Studies in thyroid transplantation. I. Data relative to the problem of secretory nerves, *Proc. Soc. Exp. Biol. and Med.*, 1914-15, xii, 202.
- Müller, Kretinismus, *Deutsch. med. Woch.*, 1914, xl, 989.
- Murphy, Jas. B., Factors of resistance to heteroplasmic tissue-grafting, *J. Exp. Med.*, 1914, xix, 513.

- Payr, E., Transplantation von Schilddrüsengewebe in die Milz; experimentelle und klinische Beiträge, *Arch. klin. Chir.*, 1906, lxxx, 730.
- Payr, E., Zur Frage der Schilddrüsentransplantation, *Arch. klin. Chir.*, 1915, cvi, 16.
- Salzer, H., Zur Frage der Schilddrüsentransplantation, *Arch. klin. Chir.*, 1909, lxxxix, 881.
- Sauerbruch, F., and Heyde, M., Ueber Parabiose künstlich vereinigter Warmblüter, *Münch. med. Woch.*, 1908, lv, 153.
- Schaack, Schilddrüsenimplantationen in das Knochenmark oder Unterhautfettgewebe, *Deutsch. med. Woch.*, 1914, xl, 989.
- Schöne, Transplantationsversuche mit artgleichen und artfremden Geweben, *Deutsch. med. Woch.*, 1911, xxxvii, 908.
- Stich, R., Ueber den heutigen Stand der Organtransplantationen, *Deutsch. med. Woch.*, 1913, xxxix, 1865.
- Stich, R., and Makkas, M., Zur Transplantation der Schilddrüse mittels Gefäßnaht, *Beitr. klin. Chir.*, 1908, lx, 431.
- Stieda, Einpflanzung von Schilddrüsengewebe, *Deutsch. med. Woch.*, 1914, xl, 988.
- Ullman, E., Tissue and organ transplantation, *Ann. Surg.*, 1914, lx, 195.
- Worobjew, W. N., and Perimow, W. A., Ein Fall von angeborenem familiärem Myxödem. Versuch von Transplantation der Schilddrüse, *Zentr. Chir.*, 1914, xli, 920.

EXPLANATION OF PLATES.

PLATE 4.

FIG. 1. Photomicrograph of intact left thyroid of Dog 2. $\times 70$.

FIG. 2. Photomicrograph of transplanted right thyroid of Dog 2 (autotransplant). Compare with the control in Fig. 1. $\times 70$.

PLATE 5.

FIG. 3. Photomicrograph of intact right thyroid of Dog 3. $\times 70$.

FIG. 4. Photomicrograph of transplanted left thyroid of Dog 3 (autotransplant). Compare with the control in Fig. 3. $\times 70$.

PLATE 6.

FIG. 5. Photomicrograph of transplanted thyroid of Dog 9 (homotransplant). $\times 70$

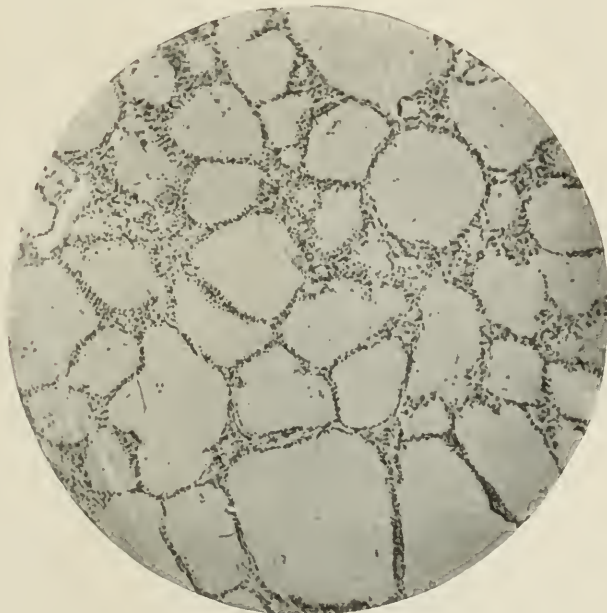


FIG. 1.

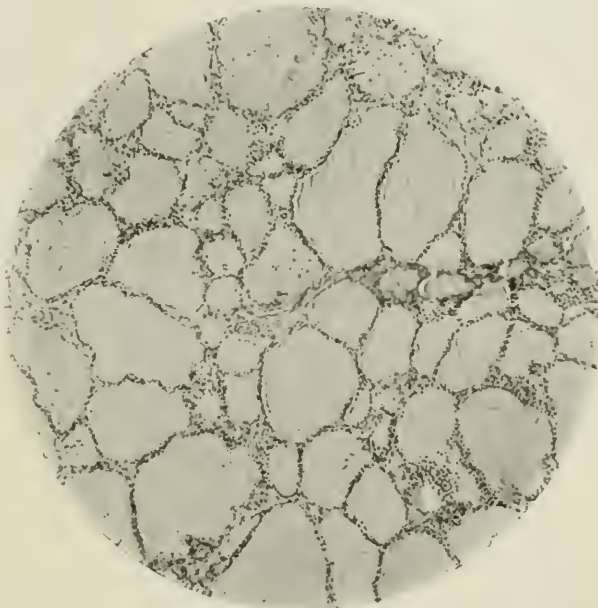


FIG. 2.

(Kawamura: Organ transplantation. I.)

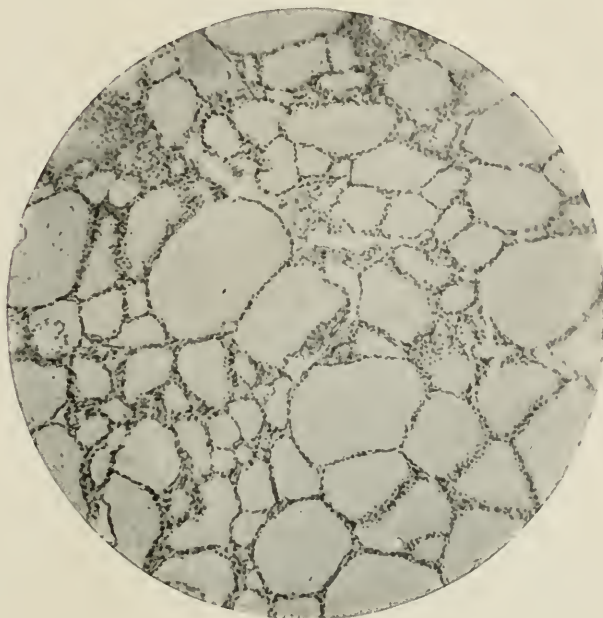


FIG. 3.

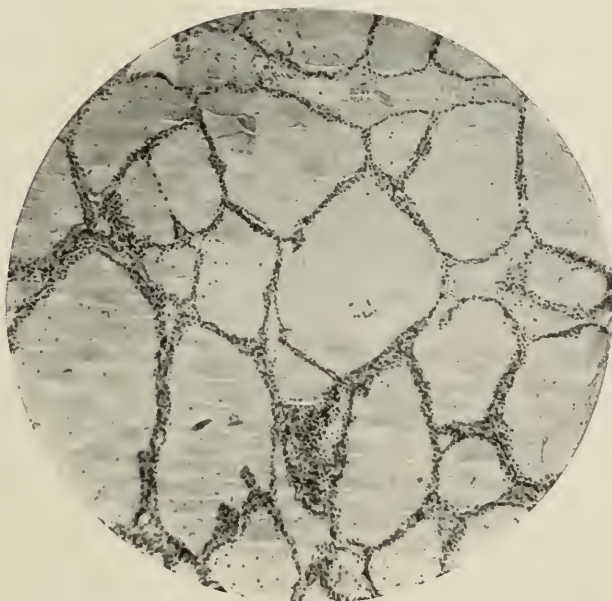


FIG. 4.

(Kawamura: Organ transplantation. I.)

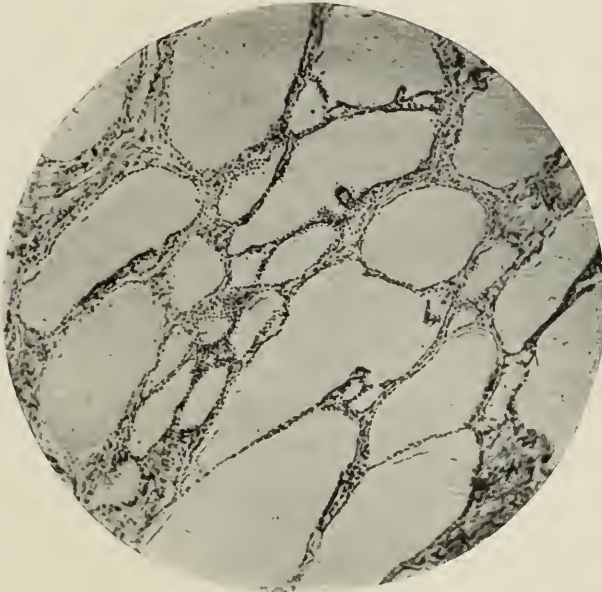


FIG. 5.

(Kawamura: Organ transplantation. I.)

STUDIES ON ORGAN TRANSPLANTATION.

II. TRANSPLANTATION OF THE SPLEEN WITH INTACT BLOOD SUPPLY.

BY K. KAWAMURA, M.D.

(*From the Division of Experimental Surgery of the Mayo Clinic, Rochester, Minn.*)

PLATES 7 AND 8.

(Received for publication, April 4, 1919.)

During the last 15 years organ transplantation has been studied extensively with especial reference to practical therapeutics and the biologic possibility of regeneration of transplanted tissues.

Many experiments have been reported on the ductless glands, and it has been affirmed that in these experiments the healing of the grafts depends largely on the site of the transplantation, and on the size of the transplant. As to the location of the graft, the opinions of authors differ widely. Cristiani, Ribbert, and others believe that the subcutaneous connective tissue is the best soil for the transplantation of the thyroid tissue; Payr preferred the spleen, while Kocher used the bone marrow. With regard to the size of the transplanting piece Cristiani, Ribbert, and Lubarsch claimed that numerous small fragments of thyroid tissue, the size of a wheat grain, are preferable. Payr, Kocher, von Bramann, and others advised the use of large grafts the size of a hazelnut or cherry because the larger portions undergo destruction.

Many instances of transplantation of the thyroid, parathyroid, ovary, and adrenals are found in the literature, and in some the results were favorable, although the percentage of success was small. On the other hand, reports of the transplantation of the spleen are rare, and the results have been unsatisfactory compared with those of the other ductless glands.

In 1916 Manley and Marine published an article on the transplantation of ductless glands. They transplanted six autografts and eighteen homografts of splenic tissue in the subcutaneous tissue of the abdomen, all of which were absorbed in 12 days. In 1917 they published another article on spleen transplantation. This time twelve attempts at homotransplantation and six attempts at autotransplantation were made on fifteen rabbits. The method employed was the same that was formerly used by them, and consisted of transferring small sections of the spleen of about 2 mm. into the subcutaneous fascia of the abdomen. None of their attempts at homotransplantation was successful beyond the usual results persisting for 2 or 3 weeks, common to all homografts. They ob-

tained only one successful permanent autotransplantation. The graft was removed at autopsy 325 days after transplantation and showed all the morphologic characteristics of a fully differentiated and functionally active spleen. The writers stated that considerable difficulty in spleen transplantation may be due to its complex anatomic structure.

The failure of transplantation of pieces of the organ, however, is partly attributable to insufficient blood supply in consequence of which the grafts are absorbed by degrees. Besides, it should be taken into consideration that small pieces, although successfully transplanted, are not always sufficient for the deficient functions. The transplantation of the entire organ by anastomosis of its blood vessels to suitable parts of the circulatory system can yield sufficient nutrition and probably also function.

Lüdke tried to transplant the spleen of dogs into the splenic vessels of a goat and a wether by blood vessel anastomosis. In all three instances there was faulty blood vessel anastomosis. He attributed the failure in the use of the technique to the unfavorable location of the spleen of these animals, and to the fact that the lumen of splenic vessels is too small. Lüdke, therefore, changed his first plan and transplanted pieces of spleen of rabbits or dogs into the abdominal cavity, the stomach wall, or into the spleen of rabbits, dogs, monkeys, goats, or wethers. The transplanted splenic tissue was observed for 4 weeks, but after 2 or 3 months no part of it was visible.

Carrel extirpated the spleen, washed it out with Locke's solution, and replaced it by blood vessel anastomosis in two dogs. One spleen necrosed and completely disappeared, due to the occlusion of the artery. The replantation of another spleen was successful, so that the anatomic condition of the vessels and of the organs was entirely normal about 8 months after the operation.

EXPERIMENTAL.

The entire series of our experiments was performed on dogs. There are usually two trunks of both artery and vein to the spleen from the gastrosplenic vessels. One pair enters the spleen in its lesser (left) end and another almost in the middle. The spleen was divided in two parts, corresponding to the stream district of these large branches, after the mattress sutures had been applied transversely on it. The half of the spleen which is nourished by the larger branches was used for the transplantation. The attached omentum was cut off after ligation. The splenic artery, vein, and nerves were dissected and

divided, Crile clamps being used. The caliber of the artery was hardly 1.5 mm. in diameter. The spleen was then removed, and wrapped up in a salt sponge. After a few minutes the spleen was replaced into the abdominal cavity, and its vessels were united as before, by end to end anastomosis. In one case an attempt was made to transplant the spleen into the neck. After the thyroid had been removed, the peripheral end of the splenic artery was united to the central end of the superior thyroid artery and the peripheral end of the splenic vein to the central end of the external jugular vein. In another case the spleen was transplanted into the renal vessels after nephrectomy. Most of the experiments were performed autoplastically, but in one instance the spleen from one animal was transplanted to another. The arterial suture was always difficult, due to the small caliber of the vessel. As soon as the clamps were unfastened, the bluish red collapsed spleen became very red, and its volume was increased. The circulation in the spleen was reestablished between 1 and 2 hours after its extirpation. The dissected omentum was reunited, and in a few cases the nerves also were sutured. Several days after the operation the condition of the transplanted spleen was ascertained by laparotomy. When the transplantation was successful, the other intact half of the spleen was removed and immersed in a jar filled with 10 per cent formaldehyde solution for microscopic examination, and the animal was observed further.

The protocol of a successful experiment is given below.

Experiment 1 (Dog 1).—Adult, mongrel bulldog, male; weight 16.9 kilos.

Oct. 5, 1918. Operated on under intratracheal ether narcosis. The spleen was exposed through a left rectus incision. Two rows of mattress sutures were placed in the middle of the spleen and the organ was divided between them. One half of the spleen, which was nourished with smaller branches of the splenic artery, was left in the abdominal cavity; the other half was used for replantation. After the dissection of the nerves and omentum, the artery and veins were cut so that this half of the spleen could be brought out extraperitoneally, apart from its connection. The spleen was wrapped up in a salt sponge for a few minutes and replanted into its original site by end to end blood vessel sutures. The arterial lumen measured hardly 1.5 mm. in diameter, and suturing was difficult. The venous suture, on the contrary, was easy. The clamp on the vein was first unfastened and then the clamp on the artery. The spleen, which during the operation appeared dark blue, and was contracted, became normal in color, and as-

TABLE I.
Summary of Experiments.

Experiment No.	Operation.	Duration of observation.	Results.			Remarks.
			Condition of blood vessels.	Condition of transplanted spleen.	Microscopic examination.	
1 (Dog 1).	1918 Oct. 5. Replantation of half of spleen. No suture of nerves.	days 88	Arterial and venous suture in perfect condition. No thrombosis.	Capsule of spleen quite thick. Normal in color and consistency. Surface of section appeared normal.	Shown features of perfectly normal spleen.	Dec. 27. Dog fought with another dog and was severely wounded in several places.
	Nov. 27. Exploratory laparotomy. Extirpation of other intact half of spleen.					Jan. 1, 1919. The wounds proved fatal.
2 (" 2).	Oct. 7. Spleen which was divided in a proportion of one-third and two-thirds by mattress suture re-planted, combined with nerve suture.	3	Thrombosis in central part of arterial suture. A small part at venous suture leaked.	Necrotic; softened.		Oct. 10. Died. A great deal of bloody fluid filled abdominal cavity.
3 (" 3).	Oct. 11. Dividing and re-planting of the spleen. Reunion of omentum and nerves.	4	Blood vessels macerated.	" "		Oct. 15. Died. A great deal of bloody serous fluid in abdominal cavity.
4 (" 4).	Oct. 12. After removal of half of the spleen, half of spleen of Dog 9 was transplanted to this dog.	2	Transplanted splenic vein thrombosed.	" "		Oct. 14. Died. Hemorrhagic serous fluid in abdominal cavity.

5 (Dog 5).	Oct. 16. After removal of left thyroid gland, spleen transplanted into neck. Splenic vein united to thyroid and splenic vein internal jugular.	Circulation through spleen very good, but volume of spleen became much larger than before transplantation. After operation animal weakened by degrees and died Oct. 23. Died during last operation; very emaciated.
6 (" 6).	Oct. 23. Replantation of half of spleen. Nov. 27. At exploratory laparotomy a large tumor was found. Jan. 24, 1919. Extirpation of tumor.	
7 (" 7).	Oct. 28. Replantation of half of spleen and removal of cut end of om and splenic nerves. Nov. 27. Exploratory laparotomy. Excision of small piece for microscopic examination.	Dec. 23. Animal looked ill. Killed by cutting of femoral artery under general ether narcosis.

CORRECTION.

On page 68, Vol. xxvii, No. 1, January 1, 1918, the formula $x = \frac{v-y}{ay}$ should read $x = \frac{ay}{v-y}$.

TABLE I—*Concluded.*

Experiment No.	Operation.	Duration of observation.	Results.			Remarks.
			Condition of blood vessels.	Condition of transplanted spleen.	Microscopic examination.	
8 (Dog 8).	1918					
	Nov. 25. After nephrectomy on left side half of spleen was transplanted to renal vessels. Dec. 9. Exploratory laparotomy. From transplanted spleen a small piece was cut off for microscopic examination. Jan. 25, 1919. Exploratory laparotomy.	days 61	Obstruction of artery at united part.	At second operation it was proved that the transplanted spleen kept its original size, but by incision no fresh blood flowed out. At third operation no part of spleen was visible.	Cells of capsule and subcapsular parts only were stainable.	The animal had been used also for experiments on transplantation of thyroid gland, and duodenectomy. Jan. 31, 1919. Died of intussusception in jejunum.

sumed its original size as soon as the clamps were removed. The circulation through the replanted spleen was reestablished after an interruption of 1 hour. No suture of the omentum or the nerves was made. After the circulation through the transplanted vessels as well as the spleen was assured, the abdominal wound was closed. The first incision was made at 9.14 a.m.; the last stitch was inserted at 11.20 a.m.

Nov. 27 (53 days after the first operation). An exploratory laparotomy was done. The weight of the animal was 17.7 kilos.; there were no marked adhesions; the omentum was normal. The replaced spleen had kept its original size and appeared entirely normal in color and consistency, but its capsule was thickened. The other intact half of the spleen was extirpated.

Dec. 27. The animal fought with another dog and was seriously wounded in the abdominal wall, back, and both hip regions.

Jan. 1, 1919 (88 days after the first operation). The dog died of his wounds.

Autopsy.—The wounds that had been made by fighting were found to be infected; there was no infection of the abdominal cavity. The replaced spleen looked very well. No thrombosis could be found in the splenic artery or vein; their lumina were patent throughout. The suture lines on the vessels could be found with difficulty. They were covered with normal epithelium and were smooth.

Microscopic Examination.—The capsule was quite thick in places as compared with the control specimen. Otherwise the structure of the pulp, the number and the size of the trabeculae, and the Malpighian bodies were perfectly normal (Figs. 1 to 4).

Seven attempts at autoplasmic transplantation and one attempt at homoplasmic transplantation of the spleen were made, as shown briefly in Table I. Five of the seven autoplasmic transplants were replanted to the splenic vessels, one to the neck, and one to the renal vessels. In two of the five replanted cases the dogs died in 3 and 4 days after the operation. At autopsy it was found that a great deal of bloody serous fluid filled the abdominal cavity, and that the spleen was necrosed, due to thrombosis. Two cases were examined 30 and 35 days after the operation by exploratory laparotomy. One half of the spleen was found intact in each case, but the other replaced half could not be seen. In one of these two instances there was about 1 liter of pale red, muddy, serous fluid in the abdominal cavity. A round, partly soft, partly hard cystic lump about the size of a child's head lay in the center of the cavity. This lump was surrounded by vast distended blood vessels and muddy gelatinous membrane. Later it was proved at autopsy that the lump was a cyst which had been formed by replanted spleen. The result of one operation (Exper-

ment 1), in which the spleen was replanted in a dog, was highly satisfactory. 88 days after the operation the spleen, as well as the united blood vessels, was in good condition. The spleen which had been transplanted into the neck was examined 7 days after the operation, and it was found that the whole tissue had undergone necrosis. The transplantation of the spleen into the renal vessels was troublesome, because the field of the operation was too deep and the caliber of the splenic artery was very small. Nevertheless, the immediate result of the operation was successful. As soon as the clamps were removed, the spleen became red and greatly distended. 14 days after the operation, when the exploratory laparotomy was made, the spleen retained its original size; but the color was pale red, and when it was incised dark blood came out. 47 days later the spleen had completely disappeared. The spleen which was grafted homoplastically was examined at autopsy 2 days after the operation. It was soft, dark red, and necrotic. The transplanted splenic vein was thrombosed. Hemorrhagic serous fluid filled the abdominal cavity.

DISCUSSION.

The results of the experiments immediately after operation were in all cases satisfactory. In spite of the interruption of the circulation from 1 to 2 hours after extirpation, the immediate circulation of blood through the transplanted spleen and blood vessels was favorable, but most of the spleen became necrotic or was entirely absorbed. The cause of this was the obstruction in the transplanted vessels, due to thrombosis. It is probably difficult to obtain good results by using so small a vessel as a branch of the splenic artery. Carrel has noted that such a small vessel cannot be sutured with many chances of success. As has been stated, all Lüdke's experiments failed.

Regardless of these difficulties, our successful case showed that such a highly differentiated, complicated organ as the spleen can be transplanted *en masse*, that it can keep permanently its normal structure, and probably also can functionate normally. In this instance the difference between the central and peripheral parts of the grafts, as Manley and Marine experienced by piecemeal transplantation, was not visible. This is scarcely to be expected because in trans-

planting by blood vessel sutures the nourishment of the transplant is maintained throughout.

In view of the fact that the spleen can survive even if the nerves are not united, the experiment demonstrated that nerves are not essential for the maintenance of grafts.

The neck, probably also the inguinal furrow, is not a favorable site for the experimental transplantation of the spleen by blood vessel suture, because after the suture of the fascia and skin the more or less distended spleen is compressed, and, consequently, disturbances of the circulation through the graft may occur.

SUMMARY.

Seven autoplasmic transplantations and one homoplasmic transplantation of the spleen of dogs were made. One autotransplantation was successful, the gland being normal at the end of 88 days.

BIBLIOGRAPHY.

- von Bramann, Ueber Schilddrüsenimplantation bei Myxödem und Kretinismus, *Deutsch. med. Woch.*, 1909, xxxv, 1738.
- Carrel, A., Remote results of the replantation of the kidney and the spleen, *J. Exp. Med.*, 1910, xii, 146.
- Cristiani, quoted by Lüdke.
- Kocher, T., Ueber die Bedingungen erfolgreicher Schilddrüsen transplantation beim Menschen, *Arch. klin. Chir.*, 1914, cv, 832.
- Lubarsch, quoted by Lüdke.
- Lüdke, H., Ueber Milztransplantationen, *Münch. med. Woch.*, 1909, lvi, 1469, 1539.
- Manley, O. T., and Marine, D., The transplantation of ductless glands with reference to permanence and function, *J. Am. Med. Assn.*, 1916, lxxvii, 260.
- Manley, O. T., and Marine, D., The transplantation of splenic tissue into the subcutaneous fascia of the abdomen in rabbits, *J. Exp. Med.*, 1917, xxv, 619.
- Payr, E., Zur Frage der Schilddrüsen transplantation, *Arch. klin. Chir.*, 1914, cvi, 16.
- Ribbert, quoted by Lüdke.

EXPLANATION OF PLATES.

PLATE 7.

FIG. 1. Photomicrograph of the intact portion of the spleen of Dog 1. $\times 50$.

FIG. 2. Photomicrograph of the same specimen as Fig. 1. $\times 125$.

PLATE 8.

FIG. 3. Photomicrograph of the transplanted portion of the spleen of Dog 1.
 $\times 50$.

FIG. 4. Photomicrograph of the same specimen as Fig. 3. $\times 125$.

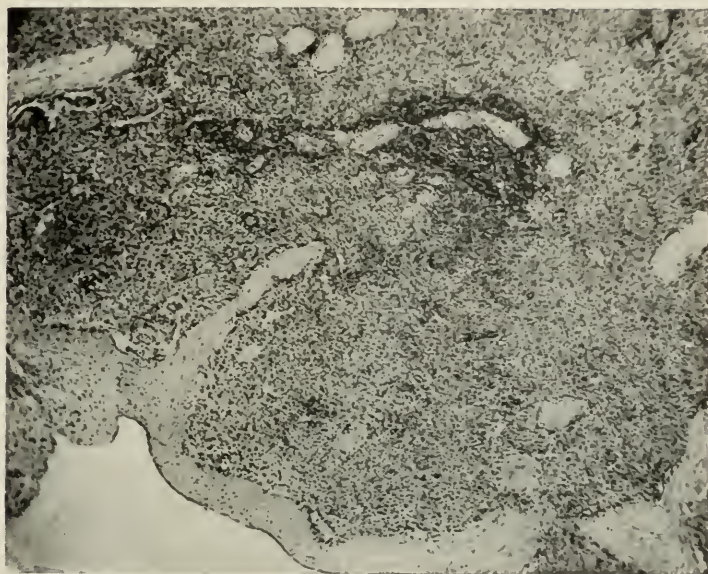


FIG. 1.

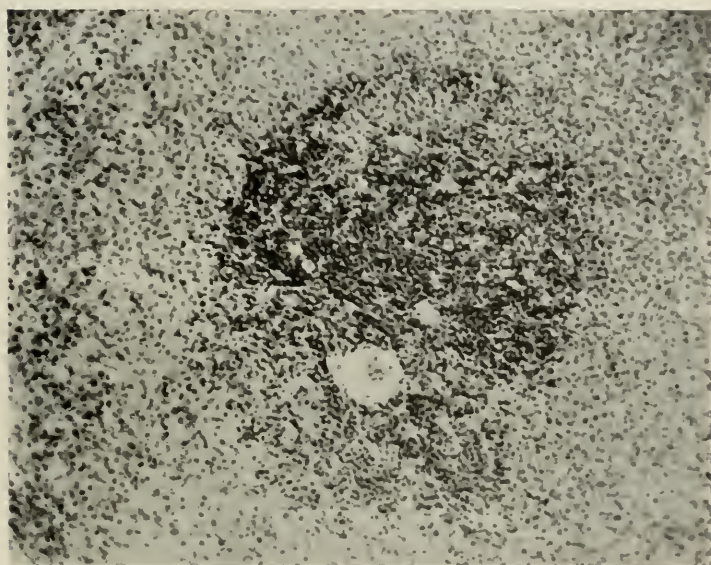


FIG. 2.

(Kawamura: Organ transplantation. II.)

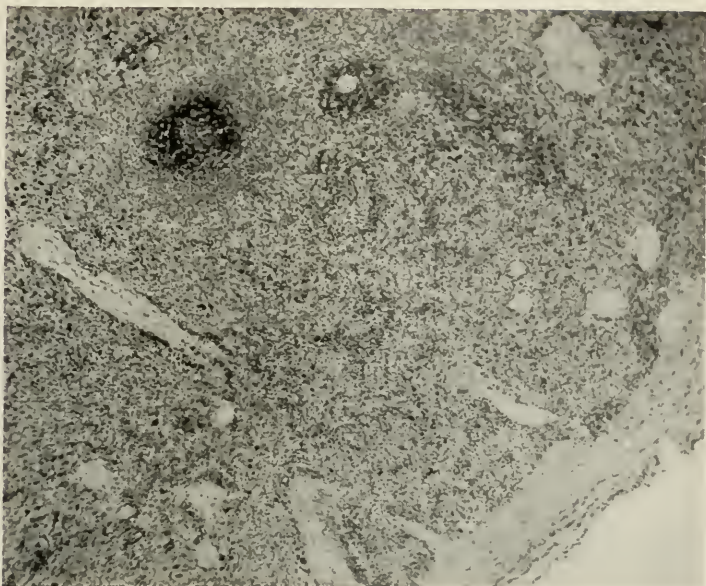


FIG. 3.

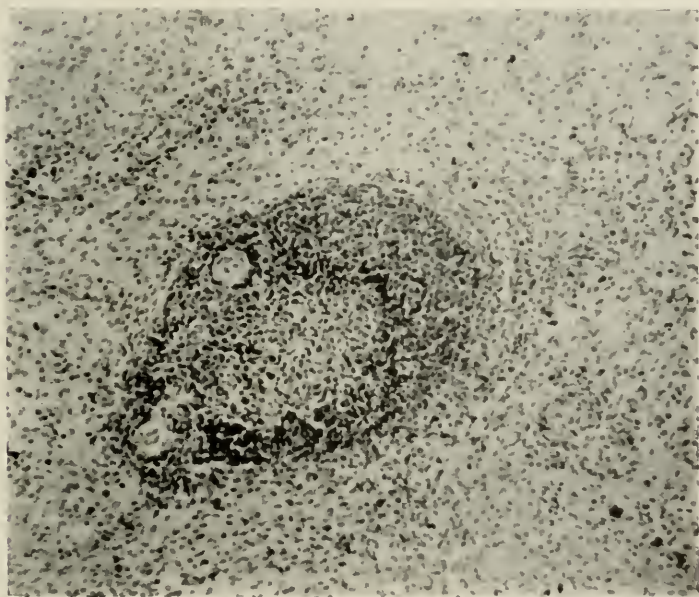


FIG. 4.

(Kawamura: Organ transplantation, II.)

THE EXPERIMENTAL PRODUCTION IN DOGS OF EMPHYSEMA WITH ASSOCIATED ASTHMATIC SYNDROME BY MEANS OF AN INTRA-TRACHEAL BALL VALVE.

BY WILLAM H. HARRIS, M.D., AND FELIX P. CHILLINGWORTH, M.D.

(From the Department of Physiology and the Department of Pathology, Tulane University of Louisiana, New Orleans.)

PLATES 9 TO 11.

(Received for publication, April 8, 1919.)

While performing certain work upon dogs with a plethysmograph devised by Chillingworth and Hopkins,¹ varying degrees of emphysema of the lungs were noted by them when the animals were autopsied. The plethysmograph was so constructed that the animal's entire body was subjected to a negative pressure by rarefying the air in the interior. The trachea was, however, connected to a tube leading to the outside air, so that the intratracheal and intrapulmonic pressure was that of the atmosphere and so much in excess of that of the extrathoracic pressure within the plethysmograph that the lungs and thorax were distended. The consequent rupture of the alveolar walls was controlled by the differences in these pressures which could be adjusted to a nicety.

The emphysema produced by this negative pressure method was acute and afforded no opportunity for clinical study, nor did this method of production simulate the factors concerned in pulmonary emphysema in man. In order, therefore, to produce a more gradual emphysema and at the same time to simulate more closely the mechanism by which this condition is brought about in man, the writers undertook to devise a method which would accomplish this purpose. Since in cases of emphysema and asthma the respiratory difficulty is mainly one of expiration, our efforts were directed to a

¹ Chillingworth, F. P., and Hopkins, R., *J. Lab. and Clin. Med.*, 1919, iv, No. 9 (in press).

duplication of this condition of altered respiration with a view to determining whether emphysema could be produced. It was found possible to accomplish this by introducing a ball valve into the trachea, which would prolong and partially obstruct expiration without impeding inspiration.

A series of valves was constructed which offered varying degrees of expiratory impediment. In the early experiments it was found that many of the valves occasioned either too great or too little expiratory obstruction. Many trials were necessary, therefore, before the correct adjustment was obtained. With properly adapted valves it has been possible to produce at will mechanical emphysema varying from acute to chronic. These valves (Fig. 1) were made of brass tubing 4 cm. long, varying in outside diameter from 8 to 13 mm. and having an inside diameter or bore of 5 to 7 mm. Upon the upper end and outer side of the valve at opposite points were soldered two narrow flat copper wings which projected about 1 cm. beyond the end of the valve. These wings when sprung slightly outward aid in maintaining the valve in position and prevent its expulsion by impinging against the mucosa of the trachea. Into each end of the cylinder a collar was inserted and these collars when so placed formed two small shelves, an upper and a lower, which projected a slight distance into the lumen of the tube. Small rectangular channels were cut into the shelves for the passage of air. The number and size of the channels were graded according to the air obstruction desired. A gutta-percha ball was placed between the two collars which during inspiration was sucked to the lower valve seat leaving the lumen of the tube open above and permitting free ingress of air through the several channels in the lower collar. During expiration the ball was raised against the upper collar, closing the upper lumen of the valve, thus permitting egress of air only through the channels, the narrowness of which offered the desired obstruction. The degree of this obstruction to expiration was graded to the number and width of the openings in the upper collar which varied from four channels with a width of 1 mm. and a length of 4 mm. down to collars carrying but two channels, having a width of 2 mm. and a length of 2 mm. The lower collar carried sufficient channels to insure practically normal inspiration.

Method of Introducing the Valve.—To insert the valves, the dogs were given a moderate dose of morphine, then deeply anesthetized with ether and placed on the belly. The tongue was pulled forward with a retractor exposing the glottis; with a long pair of forceps the lower lip of the glottis was pulled forward and the valve inserted in the trachea below the vocal cords. The size of the valve employed was selected in accordance with the size of the trachea of the animal used. The valve was carried into position by a wooden probang which entered the valve lumen readily and served as a director. When the valve was placed in position the director was withdrawn, thus completing the operation. A skiagraphic print of a valve in position is shown in Fig. 2. This particular valve had been in position 20 days.

Upon recovery from the anesthetic and morphine many of the animals for the first few hours exhibit symptoms of marked tracheal irritation characterized by violent coughing and attempts to expel the valve. Within 24 hours this phase is replaced by utter lack of local irritation; in fact in chronic emphysematous animals external manual pressure upon the trachea and valve elicits little or no response. The probable explanation of this is no doubt analogous to the adaptability of man to the tracheotomy tube. There is present in both instances a primary irritation by the foreign body, but this stage is followed by a loss of reflex stimulation; the constant contact exerts a uniform degree of pressure upon the inner coat of the trachea, thereby probably producing a pressure anesthesia upon certain terminations of the laryngeal nerves. The irritation from the valve in the dog is less than that from the tracheotomy tube in man because there is less motion or friction between the cylinder and the trachea, and, again, the air inspired is first passed through its normal route and thereby filtered and warmed as contrasted with air received directly from the atmosphere with the tracheotomy tube. The bechic spasm is probably in part relieved by the coat of mucus formed below the valve.

For the purpose of studying the carbon dioxide content of the blood and alveolar air under circumstances simulating asthma,

Friedman and Jackson^{2,3} produced expiratory obstruction in dogs. The obstruction was occasioned by employing a one-way valve in conjunction with a T-tube in the trachea. The valve was arranged so that a screw attachment would control the degree of expiratory obstruction. Their experiments were conducted with dogs under anesthesia so that the valve necessarily acted for a short duration of time, with the animal breathing through the tracheal tube and in an unconscious state. With the ball valve arrangement the dogs are observed after anesthesia over prolonged periods of time and with the source of air through the upper respiratory passages as in normal dogs. In conjunction with their observations an acute emphysema of the lungs was noted by them and described by Fraser.⁴ While a certain relationship exists in the two procedures the intratracheal ball valve permits obviously of observations under distinctly different conditions.

The present study has been based upon the observation of twenty-five dogs of varying sizes, ages, and types. The period has varied from 24 hours to 3 weeks from the time of valve introduction until autopsy. A routine observation of the dogs after the first 24 to 36 hours shows the animal apparently normal with the exception of expiratory dyspnea. This dyspnea varies from a mild to a marked degree depending upon the expiratory interference offered by the valve inserted. The presence of the valve in no way interfered with the health of the animal. The appetite was normal, and in fact with dogs that were received in a poor condition an actual gain of weight often occurred, due to proper food and care. There is a gradual enlargement and prominence of the thoracic cavity. In due course of time percussion elicits a hyperresonant note and the various features of emphysema are noted. While it might appear that accumulations of mucus would interfere with the valve action, such in reality does not occur because of the forced type of valve expiration. The valve action is readily heard with the stethoscope over the trachea. The most striking observation is the marked expiratory dyspnea noted after exercise or upon the brief inhalation of such

² Friedman, E. D., and Jackson, H. C., *Arch. Int. Med.*, 1917, xix, 767.

³ Friedman, E. D., and Jackson, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 73.

⁴ See Friedman and Jackson,² p. 768.

mild respiratory irritants as dilute ammonia fumes or tobacco smoke. The manifestations simulate closely those of asthmatic paroxysms in man even to the occurrence of the wheezing sounds.

Professor George S. Bel of the Department of Medicine was requested to examine one of the animals during a paroxysm occasioned by mild exercise. No information of any sort was given him and the following was the result of his examination: On inspection, the head was pushed forward, the thorax distended, and slight cyanosis was observed in the tongue and gums. The respirations were slow with marked prolongation of the expiratory phase. Percussion elicited a hyperresonant note over the lung area of both sides. Upon auscultation, sonorous and sibilant râles were heard over both lungs. The expiratory sounds were markedly prolonged and accompanied with wheezing. The heart was irregular and there was noted an accentuation of the second sound over the pulmonic area. Diagnosis: Marked emphysema with accompanying asthmatic attack.

In connection with other observations graphic records of respiration were carried out. The pneumograph of Ellis was employed in conjunction with a modified Marey tambour, the transmitting tube of which was sufficiently rigid to withstand all possible pressure variations. The records were found practically consistent in their curve, the down-stroke recording inspiration and the up-stroke expiration. The series of respiratory tracings shown in Figs. 3 and 4 demonstrate typically the loss of respiratory balance concomitant with pulmonary emphysema. In Fig. 5 is seen the normal respiratory curve recorded during rest, the rate being fourteen per minute. The passive type of normal expiration is clearly shown in this tracing. Fig. 3 is a record of valve respiration made after the valve had been in position 48 hours, with the animal at rest, the rate being ten per minute. While the rate of respiration is slightly slower than that in Fig. 5, the depth has been relatively increased so that this change is of no consequence. The striking difference between the normal respiration and the valve respiration as shown by the records is the increased length of time of expiration caused by the ball valve's changing the expiratory phase of respiration from passive to non-passive. Fig. 4 of this series was recorded after mild exercise which consisted in running the dog about for a few minutes.

This tracing exhibits several interesting new phases. In the first place the rate of respiration has not been increased; if anything it is slower than in Fig. 3. Normally, exercise increases the rate and depth of pulmonary ventilation, but with the valve in the trachea the respiratory rate does not increase with exercise; in fact it is mechanically slowed, but correspondingly increased in depth. After exercise the expiration is markedly forced and prolonged, and it would seem that the forced expiration is responsible for the decrease in the rate. In a few instances attacks were precipitated by the temporary inhalation of dilute ammonia fumes or tobacco smoke, and in these cases the asthma-like paroxysms were extreme. The mechanical interference with expiration results in a change in the nervous regulation which more than counterbalances the chemical stimulation from exercise. The points marked x in Figs. 3 and 4 indicate the impinging of the ball against the upper seat and it can be readily seen that the remainder of the expiratory phase which occurs between this point and the beginning of inspiration is prolonged and forced. The inspiratory act is delayed and inhibited by the slow deflation of the lungs until sufficient collapse has taken place to produce the necessary stimulation of the pulmonary branches of the vagus.

Pathology.—All the animals were autopsied and the lesions produced observed, with special reference, however, to the lungs. As death was produced in the animals by anesthesia and possible changes might occur in the lungs as a result of this, certain precautions were carried out in order to reduce to a minimum the respiratory struggle. In certain instances death was produced by toxic doses of nicotine, but as a routine procedure it was our custom to give a large dose of morphine, and after 2 hours death was produced by a small amount of ether. Morphine has the double value in that it not only prepares the animal for ether, but it also causes a marked slowing of respiratory movements, thereby minimizing distention of the alveoli which might accompany death from ether anesthesia. The gross appearance of the experimental lungs presented in general the common picture observed in human emphysema. Its production was found to vary in extent and degree according to the type of valve employed and the duration of the experiment.

Upon opening the thoracic cavity the lungs *in situ* were distended and showed no tendency to collapse. The organs were usually quite pale in color indicating a diminution of the blood supply to the parts. After removal of the lungs distinct vesicular wall rupture was noted as evidenced by numerous bullæ-like clusters present over the surface but most marked along the lung borders. Fig. 6 demonstrates the ordinary gross aspect of the pluck wherein the period of time was 7 days. In animals where the experiment extended over a short period and in which the valve employed was markedly obstructive, the emphysematous tissue was not of the pale color noted in experiments of longer duration and with less obstructive valves. In these short period experiments small areas showing hemorrhagic extravasations were occasionally noted. Such extravasations were attributed to rhexis of the smaller vessels due to the intense pressure occasioned by the alveolar distention and consequent vascular disturbance. Similar hemorrhagic extravasations were noted by Fraser⁴ in the experiments of Friedman and Jackson,² wherein an acute emphysema had been produced by their method. Occasionally at autopsy the experimentally produced emphysematous lung revealed discrete and sharply defined small consolidated areas, distinctly red in color, which proved to be secondary lobular pneumonia.

The trachea showed but slight if any pathological changes in the area from which the valve was removed. At the locations of the wings or projections which retained the valve in position varying degrees of inflammation from intense congestion to pressure necrosis of the surface with the presence of a slight amount of fibrinopurulent exudate were noted. This lesion was superficial and at no time did extensive sloughing occur. In the trachea below the valve and in the larger bronchi a slight amount of glary mucus was usually present.

Microscopic examination revealed the usual histopathology of emphysema in all the lungs studied. The walls of the air cells were abnormally thin. The interalveolar septa were in many instances ruptured giving rise to intercommunicating compartments of a few to many air cells. The larger vessels of the interstitial tissue were engorged as a result of the capillary obstruction. No evidences of

hyperplasia of connective tissue were noted. Fig. 7 is representative of the usual histopathological picture presented.

Weigert's elastic tissue stain demonstrated the ruptured fibrils. In some areas these were greatly attenuated and broken, while in others coarser fibrils were abruptly ruptured and the ends curled.

Control Animals.—As a foreign body *per se* in the trachea might have been considered sufficient to produce the results obtained, animals in which a false valve was inserted were observed and studied. The control valve differed from the others only in that its inner collar was not slotted and that it contained no gutta-percha ball. These animals after primary local irritation had passed were normal in every respect and could not be differentiated from dogs in which no valve had been introduced. They could growl and bark at will. Infrequently, a slight movement of the control valve in the trachea following marked exercise would cause a mild degree of coughing and an attempt to expel the valve. Autopsy of such control animals showed no experimental emphysema. Here and there on the lung edges slight emphysema was noted. In this connection, however, it must be stated that such a picture is observed in a considerable proportion of dogs employed in routine physiological laboratory work, especially if death has been produced by a general anesthetic.

DISCUSSION.

Emphysema in man is commonly caused by various conditions wherein there is an increase in the amount of residual air and some interference of the proper expiratory air egress. These factors may be permanent or they may be occupational or habitual. For example, emphysema is produced in those whose occupation is the blowing of wind instruments or glass blowing and the like. In these instances very full inspirations occur which include the complemental intake and are accompanied by difficult prolonged expiratory output through the instrument employed.

In certain bronchial conditions with proliferative or exudative thickening of the bronchial tubes and consequent narrowing of their lumina, it is probable that the condition may cause little if any impediment to the intake but may result in marked expiratory ob-

struction and thereby produce emphysema. The explanation of this apparent anomaly is to be found in the well known physics of respiration which have already served to explain the difficult expiration of asthma. This mechanism is clearly expressed by Starling⁵ as follows:

"There is, however, a difference in the mechanical conditions of the bronchi during the two phases of a respiratory movement. Normally the elastic structure of the lungs is drawing upon the bronchial wall, tending to maintain it patent, and so opposing the action of the bronchial muscle. During inspiration this expanding force is increased, so that in the presence of bronchial constriction the access of air is rendered the easier, the more powerful the contraction of the inspiratory muscles. In expiration all parts of the lung collapse, drawing with them the chest-wall; the pull of the lung tissue on the bronchial wall is lessened, but is still present. If, however, the expiratory muscles contract vigorously, the intrapleural pressure becomes positive, and the pull of the lung tissue on the bronchial walls is changed into a pressure tending to obliterate their lumen and so impede the out flow of air."

In addition to the above factors, it should also be remembered that at the beginning of the inspiratory act there is a rarefaction of the remaining air in the alveoli, which facilitates the ingress of air into the lungs, whereas the expiratory act is directed against atmospheric pressure.

The coughing associated with chronic bronchial lesions would seem to be a logical contributory cause in the production of emphysema just as in whooping cough. In coughing, complementary air is inspired and forcible expirations are impeded by a narrowed opening of the glottis.

The occurrence of asthma-like attacks following exercise of the animals is noteworthy, especially as asthma and emphysema are so often associated in man. It would seem that in instances wherein prolonged interference with expiration exists and under certain circumstances which demand a greater oxygen intake or carbon dioxide discharge, asthmatic seizures are precipitated. In support of this view Hoover and Taylor,⁶ Friedman and Jackson,^{2,3} and others have

⁵ Starling, E. H., Principles of human physiology, London, 2nd edition, 1915, 1048.

⁶ Hoover, C. F., and Taylor, L., *Arch. Int. Med.*, 1915, xv, 1.

shown that such attacks are precipitated by an undue amount of alveolar carbon dioxide. The exact phenomena of this process seem as yet not definitely settled. In our experimental animals moderate exercise was employed to provoke the asthmatic syndrome, but in man emotions such as excitement, mucus accumulations, and other factors might provoke a similar paroxysm. With valves causing undue expiratory obstruction and consequent insufficient carbon dioxide discharge, asthma-like attacks can be produced experimentally immediately after insertion. As such a valve occasions a persistent obstruction which cannot be relaxed like the bronchial spasm, the valve must be removed or death will ensue. In man so called bronchial asthma *per se* is sometimes attributed to bronchial constriction alone, and it is considered that paroxysms can occur without, perhaps, any apparent exudation or thickening of the mucosa; however, even in these cases the spirals of Curschmann are usually present.

It has not been our purpose in this paper to consider such forms of emphysema as the senile and compensatory or that noted in experimental anaphylaxis by Auer and Lewis⁷ and others, nor was it our intention to discuss fully bronchial or neurotic asthma. Nevertheless certain data bearing upon these may be observed by means of the experimentally produced emphysema herein described.

Our observations upon the production of emphysema in the dog have led us to believe that certain factors play an important part in its causation in man which may be enumerated as follows: (1) the degree and duration of the impediment to expiration; (2) the histological structure of the alveolar walls, including the character of the capillary vessels and of the elastic fibrils especially with reference to age; (3) the degree of bronchitis established; and (4) the efficiency of cardiac action. These factors combined or in part seem to form the basic units in the production of pulmonary emphysema.

CONCLUSIONS.

1. Experimental emphysema can be produced in dogs by impeding expiration by means of a properly constructed ball valve introduced into the trachea.

⁷ Auer, J., and Lewis, P. A., *J. Exp. Med.*, 1910, xii, 151.

2. This method affords a practical means of study of various physiological, pathological, and clinical aspects of emphysema.

3. The experiments demonstrate that any lesion or factor capable of interfering sufficiently with proper air expulsion yet permitting of sufficient air intake will occasion emphysema.

4. Attacks simulating those of asthma are occasioned in experimental emphysematous animals after exercise as well as during and following inhalation of mildly irritant gases.

EXPLANATION OF PLATES.

PLATE 9.

FIG. 1. Detailed drawing of longitudinal section of valve showing upper and lower collars with channels for passage of air. The ball is seen in a neutral position between the collars; the position in inspiration and expiration is indicated by the dotted circles. On the head end of the valve are illustrated the wings which aid in maintaining the valve in position.

FIG. 2. Dog 1. Roentgenogram of valve in position in the trachea. The trachea and larynx appear as a white tubular space above the valve. The cervical vertebræ have been rotated and extended so as to demonstrate the valve and trachea. This valve had been in position 20 days.

PLATE 10.

FIG. 3. Dog 2, female. Record of valve respiration during rest, 48 hours after valve insertion. The points marked x in this tracing and in Fig. 4 indicate the impinging of the ball against the upper valve seat. Between this point and the beginning of inspiration it will be noted that the expiration is forced and prolonged. Time 5 seconds.

FIG. 4. Dog 2. Record of valve respiration immediately following mild exercise. The extreme expiratory difficulty resulting therefrom is markedly increased.

FIG. 5. Dog 2, weight 12.33 kilos. Respiratory record of normal animal at rest. Time 5 seconds.

PLATE 11.

FIG. 6. Dog 3. Photograph of pluck, illustrating gross appearance of pulmonary emphysema. Distention of lung and bullæ-like compartments can be noted. Hemorrhagic extravasations are present on the surface of one of the lobes. This experiment extended over a period of 7 days.

FIG. 7. Microphotograph of area of emphysema demonstrating thinning of alveolar walls and rupture of some of the walls forming communicating compartments. Congestion is present in the larger blood vessels. $\times 45$.

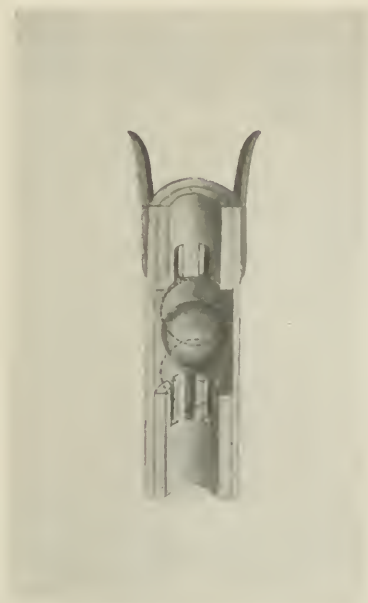


FIG. 1.



FIG. 2.

(Harris and Chillingworth: Emphysema with asthmatic syndrome in dogs.

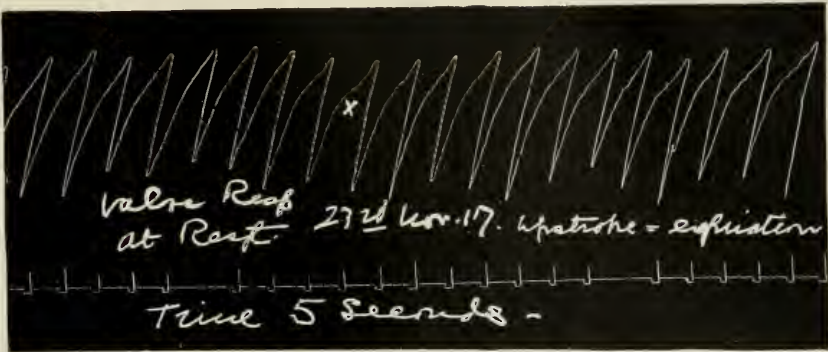


FIG. 3.

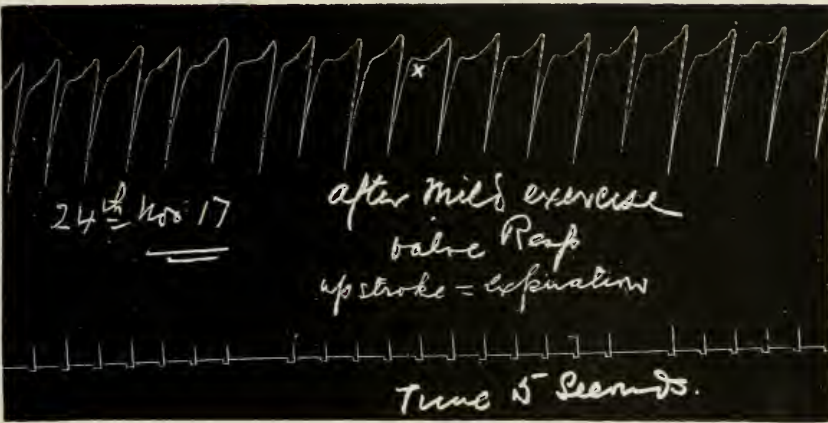


FIG. 4.

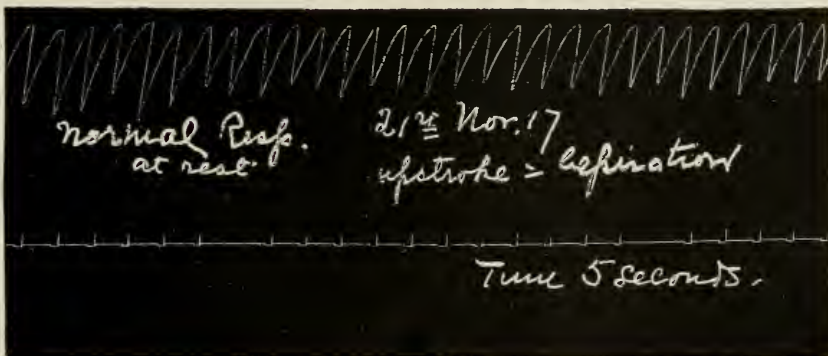


FIG. 5.

(Harris and Chillingworth: Emphysema with asthmatic syndrome in dogs.)

76



FIG. 6.

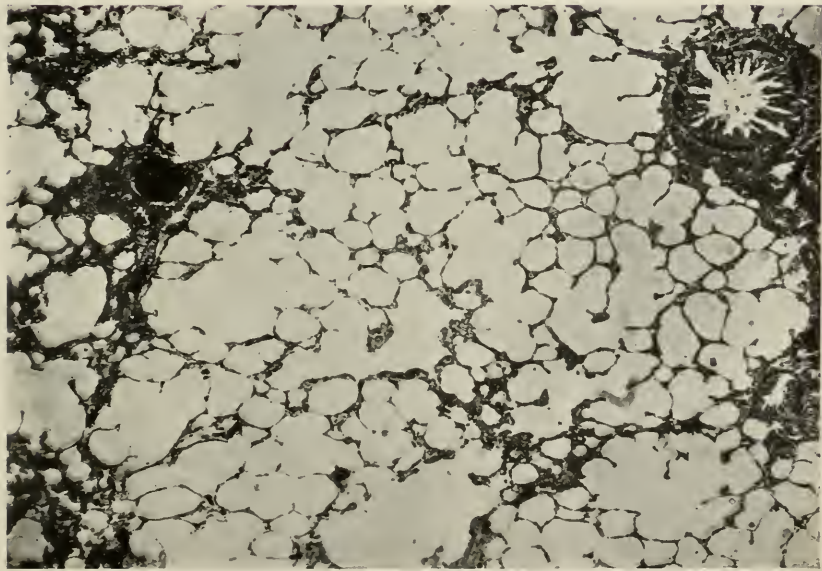


FIG. 7.

(Harris and Chillingworth: Emphysema with asthmatic syndrome in dogs).

ETIOLOGY OF YELLOW FEVER.

VII. DEMONSTRATION OF *LEPTOSPIRA ICTEROIDES* IN THE BLOOD, TISSUES, AND URINE OF YELLOW FEVER PATIENTS AND OF ANIMALS EXPERIMENTALLY INFECTED WITH THE ORGANISM.

By HIDEYO. NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 16, 1919.)

The number of *Leptospira icteroides* found in the blood of yellow fever patients was so small that a prolonged examination of blood specimens was necessary to discover one organism, even when positive transmission had been obtained by injection of the blood into guinea pigs. In most cases the organism could not be detected in the blood, perhaps owing to lack of time. To devote much time to microscopic examination during the period while the work with yellow fever patients was being carried on was impossible and also inadvisable, since it was a matter which could be taken up at a later date when more time was available. Hence, what will be reported in this paper is not final but is the preliminary account of what was accomplished under the circumstances. The demonstration of the organism in the blood and various organs of animals experimentally infected with *Leptospira icteroides*, on the other hand, was much more satisfactorily accomplished. The infection could be produced at the desired time and the material obtained in sufficient quantity at any stage of the disease.

Examination of Material from Yellow Fever Cases.

As already stated¹ a minute organism belonging to the genus *Leptospira* has been found both in the blood and in the tissues of various organs, particularly the liver and kidneys, of guinea pigs

¹ Noguchi, H., *J. Exp. Med.*, 1919, xxix, 565.

which contracted an experimental infection after being inoculated with the blood of yellow fever patients taken during the early stages of the disease. In three instances (Cases 1, 4, and 6) the organism was found in the cultures made directly from the blood of yellow fever patients. A special effort was made, therefore, whenever a case of yellow fever was admitted to the hospital or an autopsy was performed, to find the same organism in the blood and tissues, and if possible also in the urine.

The dark-field microscope was used for examination of the fresh material. Films and sections were stained by Giemsa's or Wright's stain, by Levaditi's or Noguchi's silver impregnation methods, as occasion demanded. Fontana's silver impregnation method was applied several times without satisfactory results. Blood films were usually fixed with methyl alcohol,² while the impression films of various tissues from autopsies were first fixed over osmic acid vapor for 1 minute and then in absolute alcohol for 30 minutes. They were stained with Wright's stain for 30 minutes and then with Giemsa's stain over night. Six to twelve were prepared from each specimen of blood or tissue for examination.

Of twenty-seven cases of yellow fever in only three (Cases 3, 4, and 14) was the leptospira demonstrated in the blood under the dark-field microscope, and in each instance the number of the organisms found was so small that once they had passed out of the field they were not easily encountered again. The blood of two of these three patients (Cases 3 and 4) also yielded positive transmission into guinea pigs.¹ The organism was found in a few of the stained film preparations, but these will have to be repeatedly examined. Some specimens of yellow fever blood, however, were infectious for guinea pigs even when in the fresh state no leptospira could be demonstrated under the dark-field microscope (Cases 1, 2, 5, and 6). That there must have been a very small number of the organisms in such specimens is shown by the fact that the blood and organs of the infected animals contained the organisms. A negative microscopic examination of the blood indicates either absence or scarcity of the organism. Nor does the failure of the blood to reproduce a fatal experimental

² For the purifying of the methyl alcohol for this purpose I am indebted to Dr. Herman Edward Redenbaugh of the Commission.

infection in guinea pigs prove the absence of the organism in the specimen, since variations in its pathogenicity for guinea pigs are considerable (Case 14).

Examination of various organs under the dark-field microscope has yielded so far only one positive finding, that in the liver of Patient J. Co. (Case 4), who died on the 4th day of the disease. The liver was excised from the body (a partial autopsy) a few hours after death while the body was still quite warm. The kidney failed to show any leptospira. In the stained specimens of the blood, liver, and kidney a small number of leptospiras was demonstrated. Both the blood and the liver emulsion of this patient yielded a positive transmission of the disease to guinea pigs.¹

A careful search for the organism was carried out with the films made from the liver, kidney, lungs, adrenals, mesentery, and inguinal glands from ten more cases, with so far a positive finding in the liver of one case (Case 5). Some of these slides were poorly stained and will have to be repeatedly examined when more time is available. In a later paper will be recorded the results of the examination of sections of the organs from patients who died of yellow fever.

Specimens of urine from twenty-one cases of yellow fever were examined under the dark-field microscope, but no leptospira was encountered. The examinations were made during the height of the illness, which is usually the 5th or 6th day of the disease, as well as during convalescence, towards the end of the 2nd week. This part of the investigation was much handicapped by the lack of a powerful centrifuge to concentrate the urine, and it will have to be repeated under more favorable conditions. As might be expected, *Treponema minutum*³ and *Treponema calligyrum*⁴ were occasionally seen in the samples of urine. A doubtful result was obtained in a guinea pig inoculated with 10 cc. of the urine from a convalescent (Case 51) on the 15th day of the disease, although no leptospira was found in the specimen.

³ Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 667.

⁴ Noguchi, H., *J. Exp. Med.*, 1913, xvii, 89.

Examination of Material from Experimentally Infected Animals.

Experiments were undertaken to determine the approximate time when the blood, liver, and kidney of guinea pigs infected with the yellow fever virus first contain enough organisms to produce the infection when transferred to a normal guinea pig, and when they cease to be infective. These points are more of academic interest, as they do not affect the present sanitary measures with regard to isolation of the patients before, during, and after the actual sickness.

When a minute quantity of the virus is introduced into the subcutaneous tissue of the guinea pig no local reaction follows. The blood becomes infective as early as 48 hours after the inoculation in some instances, but more constantly so after 72 hours. The liver and kidney become infective simultaneously with the blood. The amount of blood effective for infection is comparatively large, at least 1 cc. being required during the earlier period. The demonstration of the organism in the blood is almost hopeless and has not been successful until the 5th day after inoculation, when occasional specimens have been found after a long search. The organisms are more readily found in the emulsions of the liver or kidney, sometimes more numerous in one and sometimes in the other. After the onset of the disease the number of organisms gradually increases for a time, both in the blood and in the liver and kidney. Then the relation of the organism to the blood on the one hand and to the liver and kidney on the other seems to show a certain difference. The organisms continue to increase in the organs somewhat longer than in the blood. In fact, the number of organisms in the blood becomes fewer as the disease progresses, and only a few can be detected when the jaundice and other symptoms have fully developed; that is, a day or two before death. This has been the rule with the majority of the guinea pigs in the present experiments, but there were exceptions in which the organisms were quite numerous until death. Although they increase in the liver and kidney in greater number and for a longer period, they have been found in most instances finally to disappear also from these organs. The disappearance is sudden and usually occurs about 24 hours before death, although there are instances in which they can be found in large numbers at death, sometimes

more abundantly in one or the other organ. Although it is true that when the blood contains the leptospira at the time of death the liver and kidney also contain it, yet the reverse is not always the case, the organism being found in the organs but not in the blood. An instance has not been met in which the leptospira was present in the blood but not in the liver or kidney or both.

The mechanism of the disappearance of the leptospira in so many typical experimental guinea pigs during the later stage of the infection is difficult to explain, but it may be partly due to the formation of certain still undetermined metabolic products brought about by the disorganization of the liver or kidney or some other organs. The leptospira is highly sensitive to the dissolving action of bile salts *in vitro*, and it is not inconceivable that a predecessor or a derivative of taurocholic, glycocholic, or cholic radicals in a certain stage may exert a powerful destructive action upon it.

With regard to the question of the infectivity of the blood and organs in the later stage of the infection, the statement may be made that in all instances in which the organism was seen, whether in the blood or in the organs, the material was always infective. On the other hand, in a considerable number of instances in which the dark-field search for the organism failed, no infection could be induced in normal guinea pigs by inoculation of the material. It may be supposed that the organisms had completely disappeared from the body in these cases. However, as was to have been expected, there were many instances in which, notwithstanding an unsuccessful microscopic search for the organism, inoculation of the liver or kidney, or both, but not the blood, gave rise to a typical infection in further passage, with the reappearance of the leptospira in the new host. In one instance the leptospira was microscopically detected on the third passage. It may be concluded, then, that in experimental infection in the guinea pig the specific organism survives longer in some of the infected hosts than is assumed to be the case in yellow fever in man.

SUMMARY.

Examinations of fresh blood from yellow fever patients by means of the dark-field microscope, made in more than twenty-seven cases,

revealed in three cases the presence of *Leptospira icteroides*. In no instance was a large number of organisms found, a long search being required before one was encountered. The injection of the blood into guinea pigs from two of the three positive cases induced in the animals a fatal infection, while the blood from the third positive case failed to infect the guinea pigs fatally. Careful but by no means exhaustive dark-field searches for the leptospira with fresh specimens of blood from the remaining cases of yellow fever ended without positive findings, although four of the specimens, when injected into guinea pigs, caused a fatal leptospira infection.

Stained blood film preparations from the corresponding cases were also examined, but the percentage showing the leptospira in the blood was no greater than that found by examination in the fresh state with the dark-field microscope. In fact, owing to the defective stains that were available at the time of the investigation a great many slides did not take the proper coloration with Giemsa's or Wright's stain and could not be relied upon.

Regarding the presence of *Leptospira icteroides* in various organs both dark-field and stained films were examined. In only one instance so far a few organisms were detected in the emulsion of liver taken shortly after death from a case dying on the 4th day of yellow fever. This part of the work will be reported later upon completion.

Examinations of the urine from different cases of yellow fever were made both by dark-field microscope and by inoculation into guinea pigs. The results were totally negative in thirteen cases, including many convalescents, but in one case one of the guinea pigs inoculated with 10 cc. of the urine came down on the 15th day with suggestive symptoms (suspicion of jaundice, and some hemorrhagic and parenchymatous lesions of the lungs and kidneys). This specimen showed no leptospira by dark-field examination.

In experimental infection of guinea pigs with *Leptospira icteroides* the blood became infective in many instances 48 hours after inoculation, and was always infective after 72 hours. The liver and kidney become infective simultaneously with the blood. Detection of the organism by means of the dark-field microscope has seldom been accomplished before the 5th day. The organisms are most abundant on the 6th to the 7th day, but become fewer or completely

disappear before death. In the meanwhile the number of organisms increases in the liver and kidney, from which they disappear as the jaundice and other symptoms become aggravated. When death occurs these organs seem to have lost most of the leptospira, and positive transfer by means of them is less certain. At the later stage of the disease the blood is often free from the organisms and ceases to be infective. Positive transmission with blood obtained from moribund animals is not impossible, however, even when no leptospira can be detected under the dark-field microscope.

ETIOLOGY OF YELLOW FEVER.

VIII. PRESENCE OF A LEPTOSPIRA IN WILD ANIMALS IN GUAYAQUIL AND ITS RELATION TO LEPTOSPIRA ICTEROHÆMORRHAGIÆ AND LEPTOSPIRA ICTEROIDES.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 16, 1919.)

Leptospira icterohæmorrhagiæ was demonstrated in the kidneys of wild rats first by Ido and his associates in Japan,¹ then by Stokes, Ryle, and Tytler,² Courmont and Durand,³ Martin and Pettit,⁴ Renaux,⁵ Coles,⁶ Monti,⁷ Grasso,⁸ and Dalmau⁹ in Europe; and by Noguchi¹⁰ and Jobling and Eggstein¹¹ in North America; by Nicolle and Lebaillly¹² in Tunis, and by Lhéritier¹³ in Algeria. That the leptospira found in wild rats is in all probability identical with that which produces infectious jaundice in man was the conclusion of Ido and his associates¹ and later of the writer¹⁰ from the reciprocal immunity reactions between the human and rat strains. Just how the rat strain enters the human body is still a

¹ Ido, Y., Hoki, R., Ito, H., and Wani, H., *J. Exp. Med.*, 1917, xxvi, 341.

² Stokes, A., Ryle, J. A., and Tytler, W. H., *Lancet*, 1917, i, 142.

³ Courmont, J., and Durand, P., *Bull. et mém. Soc. méd. hôp. Paris*, 1917, xli, 115.

⁴ Martin, L., and Pettit, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 10, 574; 1918, lxxxi, 697.

⁵ Renaux, E., *Compt. rend. Soc. biol.*, 1917, lxxx, 405.

⁶ Coles, A. C., *Lancet*, 1918, i, 468.

⁷ Monti, A., *Boll. Soc. med.-chir. Pavia*, 1917, cited by Martin, L., and Pettit, A., *Spirochétose ictérohémmorrhagique*, Monographies de l'Institute Pasteur, Paris, 1919.

⁸ Grasso, G., *Pathologica*, 1918, x, 8.

⁹ Dalmau, M., *Treballs Soc. Barcelone*, 1918, cited by Martin, L., and Pettit, A., *Spirochétose ictérohémmorrhagique*, Monographies de l'Institute Pasteur, Paris, 1919.

¹⁰ Noguchi, H., *J. Exp. Med.*, 1917, xxv, 755.

¹¹ Jobling, J. W., and Eggstein, A. A., *J. Am. Med. Assn.*, 1917, lxix, 1787.

¹² Nicolle, C., and Lebaillly, C., *Compt. rend. Soc. biol.*, 1918, lxxxi, 349.

¹³ Lhéritier, A., *Bull. Soc. path. exot.*, 1918, xi, 357.

matter of conjecture, based on circumstantial evidence. It is not yet entirely clear how the leptospira happens to be carried by rats. It is probable that the infection is an accidental one, occasioned by the direct transmission of the leptospira to man through exposure of some part of the body to the water of a cess-pool or to moistened ground on which the urine of an infected rat has been deposited within a few hours previously. In a longer period the organisms would be destroyed by other saprophytic bacteria.¹⁴ The incidence of infection is much greater in countries where the body is exposed, especially the feet and hands, to ground or water infested by wild rats. The occupation of the people also plays an important part in the frequency of infection. Once a rat becomes infected it supplies the infective agent for an indefinite length of time, possibly until its death. Transmission of the leptospira from rat to rat is accomplished by infected food or drink and is particularly easy in view of their cannibalism.

The question of the presence of a leptospira in tropical countries, especially where endemic foci of yellow fever exist, has not heretofore been studied, but with the isolation of a pathogenic leptospira from certain cases of yellow fever in Guayaquil the relation between the human and animal strains demands a thorough investigation. A general survey has therefore been made to detect the presence of the leptospira among the wild animals encountered in Guayaquil.

The mode of study consisted in inoculating intraperitoneally 1 to 2 cc. of a Ringer solution emulsion of both kidneys of two animals into three guinea pigs. The emulsions were prepared, in the proportion of 1 gm. of the kidney to 10 cc. of Ringer solution, by finely grinding up the kidneys in a mortar with sterile sand. Rats, mice, bats, and an opossum were examined for the presence of the leptospira. These animals are abundant in and about the houses and buildings in Guayaquil, and they were caught and sent in alive by the Board of Health¹⁵ of the city. Table I shows the results of the investigation.

The experiments show that eight out of twelve groups of rats carried in their kidneys a leptospira capable of producing in guinea pigs pathological changes similar to those produced by *Leptospira icteohæmorrhagiæ*. Only two groups of rats were absolutely free

¹⁴ Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 593.

¹⁵ I am greatly indebted to Dr. León Becerra of the Board of Health, for his cooperation in the course of the work which was carried on in Guayaquil. For some specimens of bats I am also indebted to Dr. Gonzales-Rubio, Jr.

TABLE I.
Experiments with Rats.

Groups of rats.	Guinea pig No.	Results.	Remarks.
1 (doubtful).	373	Temperature 40.2°C. on 5th day. Survived.	Perhaps an abortive infection.
	374	Temperature 39.8°C. on 5th day. Survived.	“ “
	375	Apparently no reaction.	Negative.
2 (positive).	376	Temperature 40.4°C. on 6th day. Epistaxis and icterus on 7th day. Killed. Lungs and stomach hemorrhagic. Liver icteric and enlarged. Acute parenchymatous nephritis. Other organs unchanged. No leptospira found by dark-field examination.	Transfer to Guinea Pig 377 A (blood 2 cc.). Temperature 40°C. on 5th day. Killed “ 8th “ Typical, but no leptospira found by dark-field examination.
	376 A	Apparently no febrile reaction. Animal became icteric with temperature of 36.2°C. on 8th day. Killed. Findings typical; difficult to find leptospira in blood or organs.	In a later passage the leptospira was demonstrated.
	377	Died over night.	
3 “	378	Only slight temperature rise, but typically jaundiced on 8th day and was killed. Typical changes of the organs. Leptospira difficult to find.	
	338 A	Died in 9 days without typical changes.	
	389	Died over night.	
4 “	380	Became jaundiced on 5th day. Died in 9 days. Typical findings. Leptospira not demonstrated.	
	381	Temperature 40.2° on 10th day; 40°C. on 11th day, but animal never came down with typical symptoms.	
	382	Temperature 40.5°C. on 8th day and 37.4° on 10th day, with extreme jaundice. Typical findings with leptospira in the tissues.	

TABLE I—*Continued.*

Groups of rats.	Guinea pig No.	Results.	Remarks.
5 (negative).	383	Died of intercurrent infection in 10 days.	
	384	No reaction.	
	385	Temporary rise in temperature for several days. Survived.	
6 "	386	Died in 2 days. Negative.	
	386 A	Temperature 40.3°C. on 7th day, but with ultimate recovery.	
	387	No reaction.	
7 (doubtful).	388	Nothing typical.	Perhaps an abortive infection.
	388 A	Temperature 40.2°C. on 4th day, but recovered.	
	389	Died in 14 hours. Negative.	
8 (positive).	390	Temperature 40.2° on 5th day, 38.6°C. on 7th, with marked jaundice. Killed. Typical findings in the organs; leptospira not demonstrated by dark-field examination.	Transfer to two guinea pigs (blood 2 cc.). Both became typically yellow and were killed on 10th day. Typical findings, with leptospira in liver, but none in kidney or blood, of one animal; second animal showed no leptospira.
	390 A	Highest temperature 39°C. on 5th day. Died in 9 days. Typical lesions.	
	391	Slight febrile reaction. Recovered.	
9 "	392	Highest temperature 39.4°C. on 5th day. Died on 7th day with typical lesions.	
	392 A	Highest temperature 39.6°C. on 8th day. Died on 10th day with icterus and hemorrhages.	
	393	Temperature 40.1°C. on 6th day, 39.9° on 7th day. Transient icterus on 10th day. Recovered.	

TABLE I—*Concluded.*

Groups of rats.	Guinea pig No.	Results.	Remarks.
10 (positive).	394	No high fever. Icterus in 8 days. Killed in 11 days. Lesions typical. <i>Leptospira</i> difficult to find.	Transfer to two guinea pigs. Both died in 7 days with typical lesions and jaundice.
	394 A	Temperature 39.4°C. on 5th day. Icterus following day. Killed on 8th day. Typical lesions at autopsy. <i>Leptospira</i> found.	
	395	Remained well.	
11 “	396	Temperature 39.7°C. on 4th, 40° on 5th, 36.6° on 7th day. Intensely jaundiced. Killed in 6 days. Typical changes. <i>Leptospira</i> difficult to find.	Transfer to two guinea pigs (blood 2 cc. each). One died in 7 and the other in 9 days—both intensely jaundiced. Typical lesions. Melena and black vomit in latter.
	396 A	Had very little fever, but died with jaundice in 9 days. Autopsy typical.	
	397	Temperature 39.6–39.8°C. on 6th, 7th, and 8th days. Suspicion of icterus. Killed for examination in 15 days. Lungs showed old hemorrhagic areas; other organs not changed.	This animal was convalescing after a mild infection.
12 “	398	Remained well.	
	399	“ “	
	400	Died in 13 days, with typical jaundice and lesions.	

from the organism, while two others showed a suspicious reaction without terminating in fatal infection. In other words, about 67 per cent of the rats studied harbored the *leptospira* in their kidneys.

In testing mice for the same organism it was found that out of three groups of seven mice each, one produced extreme jaundice and the other typical changes in two out of three guinea pigs, one dying in 8 and the other in 9 days after the inoculation of the kidney emulsion. There was hemorrhage before death from the rectum in one

and from the nose in the other. The leptospira was difficult to find in the blood, liver, and kidney, but was found in subsequent passages.

No positive results were obtained with the kidney emulsions of eight bats and one opossum. It may be noted that Nicolle and Lebailly¹² obtained negative results with bats caught in Tunis.

The experiments show that the emulsions which produced a fatal infection in guinea pigs did not always kill all the guinea pigs inoculated with the same quantities of the same material and under the same conditions. Some died, while others had a mild abortive infection or escaped infection altogether. This seems to indicate that there exists among individual guinea pigs a considerable variation in their susceptibility to the same strain of the organism and explains why it is important to use as many guinea pigs as convenient for the purpose of transmitting the organism to this animal. There is a close analogy with the frequent abortive infections which were obtained in the attempts to transmit *Leptospira icteroides* to guinea pigs.

The relation between the rodent strains and the strains of *Leptospira icteroides* on the one hand and those of *Leptospira icterohæmorrhagiæ* on the other was next studied from the standpoint of pathogenicity and immunity. For this purpose two strains of leptospira isolated from rats and one from mice were used. The three strains, designated Groups 8, 11, and 30, have morphological features identical with those of the strains isolated from wild rats caught in the vicinity of New York,¹⁰ and are consequently indistinguishable from the icterohemorrhagic strains derived from the Japanese and European sources. They are slightly coarser than the strains of *Leptospira icteroides*.

With regard to the pathogenicity of the Guayaquil rat leptospira, it is difficult to point out any essential difference between the symptoms and lesions that occur in guinea pigs infected with it and those in animals inoculated with the icterohemorrhagic strains of temperate climates. They all produce jaundice and hemorrhages, although with some strains hemorrhage is the outstanding feature.

Identification of the Organism by Means of Immunity Reactions.

In order to determine whether the leptospira isolated from wild rodents in Guayaquil is identical with the strains of *Leptospira icterohæmorrhagiæ* from other sources, and what relation it may have to *Leptospira icteroides* from yellow fever patients in the same city, their immunity reactions were taken into consideration in a series of experiments.

Immune Sera.—Two rabbits were immunized with each of the three strains of Guayaquil rodent leptospira by injecting intravenously 2 to 4 cc. of rich living cultures of the organisms several times at 7 to 12 day intervals. The animals were bled on the 9th day after the last injection and the effects of their sera tested not only upon the same and other strains of Guayaquil origin, but also upon the Japanese, European, and New York strains of *Leptospira icterohæmorrhagiæ*. The relation of this group of organisms to that of *Leptospira icteroides* was likewise studied and will be discussed at greater length in connection with the relation between yellow fever and infectious jaundice.

The first experiments were designed for observation of the action of each of these sera upon the organism *in vitro*. To 0.5 cc. of a rich suspension of culture in saline solution was added 0.2 cc. of the immune serum with or without the simultaneous addition of 0.2 cc. of fresh normal guinea pig serum as complement. The mixture was placed in a water bath at 37°C. for 2 hours and then examined under the dark-field microscope. The entire procedure was carried out with strict aseptic precautions, and each experiment was accompanied by a control with normal rabbit serum. The tubes were kept at room temperature for 96 hours and their contents examined again. Except for a greater amount of precipitation in some tubes the results were about the same as those observed at the end of 2 hours at 37°C. The control tubes with normal rabbit serum showed numerous active organisms after 4 days.

Pfeiffer's phenomenon was also studied by the usual procedure; that is, examination of the peritoneal fluid of guinea pigs after inoculation with a given serum and the strain in question. In this instance 0.5 cc. of rich culture was mixed with 1 cc. of the immune serum and

injected intraperitoneally, examination being made after 30 minutes and 2 hours.

As another means of identification the protective property of each immune serum (1 cc.) was tested on guinea pigs against approximately 10 minimum lethal doses of the different strains. Unfortunately

TABLE II.

Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.

Immune Serum 906, produced with Group 8 strain, injected on Jan. 15, 22, Feb. 3, 15, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Marked precipitation, agglutination, and disintegration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Effects similar to the foregoing but less in degree.	"	" "
" 30	Marked agglutination and later disintegration.	"	" "
Japanese.	Similar to the foregoing but less marked.	"	" "
American No. 1	Rather marked agglutination.	"	Could not be tested because of loss of virulence.
" " 3	Effects similar to the foregoing.	"	" "
French.	" "	"	1 cc. protected guinea pigs against about 10 minimum lethal doses.

the virulence of the American and British strains of *Leptospira icterohæmorrhagiæ* was considerably attenuated during my absence of 6 months and could not be tested with reliable results. This, however, was not a serious obstacle to determining the affinity of these strains for the Guayaquil strains, because an immune serum produced in rabbits with the avirulent American strain was tested against the pathogenic Guayaquil strains. A brief summary of the foregoing experiments is given in Tables II to VI.

TABLE III.

Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.

Immune Serum 914, produced with Group 11 strain, injected on Dec. 30, 1918, Jan. 6, 14, 21, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Moderate agglutination but no degeneration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Marked precipitation and agglutination; later degeneration.	"	" "
" 30	Effects similar to the foregoing but less marked.	"	" "
Japanese.	" "	"	" "
American No. 1	" "	"	Could not be tested because of loss of virulence.
" " 3	Marked agglutination and degeneration.	"	" "
French.	" "	"	1 cc. protected guinea pigs against about 10 minimum lethal doses.

TABLE IV.

Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.

Immune Serum 904, produced with Group 30 strain, injected on Dec. 30, 1918, Jan. 6, 14, 22, Feb. 3, 15, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Marked agglutination and subsequent disintegration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Slight agglutination, but no degeneration.	"	" "
" 30	Marked precipitation, agglutination, lysis, and degeneration.	"	" "
Japanese.	" "	"	" "
American No. 1	" "	"	Could not be tested on account of loss of virulence.
" " 3	Slight agglutination; no degeneration.	"	" "
British.	" "	"	" "

TABLE V.

Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.

Immune Serum 952, produced with American Strain 1, injected on Dec. 30, 1918, Jan. 6, 14, 22, Feb. 3, 15, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Moderate agglutination; slight degeneration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Very marked agglutination and degeneration.	"	Not tested.
" 30	Slight agglutination and degeneration.	"	1 cc. protected guinea pigs against about 10 minimum lethal doses.
Japanese.	Moderate agglutination and degeneration.	"	Not tested.
American No. 1	Marked agglutination and degeneration.	"	" "
British.	Slight agglutination and degeneration.	"	" "

TABLE VI.

Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.

Immune Serum 911, produced with the Japanese strain, injected on Dec. 30, 1918, Jan. 6, 12, 22, Feb. 3, 15, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Moderate agglutination and degeneration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Slight agglutination; no degeneration.	"	" "
" 30	Marked agglutination and disintegration.	"	" "
Japanese.	Very marked agglutination and lysis.	"	" "
American No. 1	Moderate agglutination and degeneration.	"	Could not be tested because of loss of virulence.
British.	Slight agglutination and degeneration.	"	" "

The tables show that while there exist undeniable variations in the intensity of the immunity reactions as manifested *in vitro* in the form of agglutination and subsequent disintegration of the organism, as well as *in vivo* in Pfeiffer's phenomenon and protection against infection with one or the other strains, the variations are nevertheless of so slight a nature as to lead one to assume that the strains isolated

TABLE VII.

Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icteroides.

Immune Serum 906, produced with Group 8 strain, injected on Jan. 15, 22, Feb. 3, 15, 1919.

<i>Leptospira icteroides</i> strain.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
A. A.	Slight agglutination; no degeneration.	Negative.	No protection.
E. Ch.	No effect.	"	" "
A. Ce.	" "	"	" "

TABLE VIII.

Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icteroides.

Immune Serum 914, prepared with Group 11 strain, injected on Dec. 30, 1918, Jan. 6, 14, 21, 1919.

<i>Leptospira icteroides</i> strain.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
A. A.	No effect.	Negative.	No protection.
E. Ch.	" "	"	" "
A. Ce.	" "	"	" "
M. G.	Definite agglutination, but no immobilization.	Doubtful.	1 cc. did not prevent the infection, but the animal survived.

from rats and mice in Guayaquil belong to the group of *Leptospira icterohæmorrhagiæ* now known to be widely distributed among these rodents inhabiting the temperate zone.

The results of a parallel series of experiments performed with the immune sera on different strains of *Leptospira icteroides* are given in Tables VII to IX.

Prophylactic Inoculation.—February 6, 1919. A number of guinea pigs were inoculated subcutaneously with 0.5 and 2 cc. of killed culture (heated to 60°C. for 10 minutes in the waetr bath) of the Guayaquil rat strains of leptospira, Groups 8, 11, and 30. These animals were inoculated after 15 days (February 21) with virulent cultures of the same and of other strains. At the same time some of the vaccinated guinea pigs were inoculated also with the icterohemorrhagic strains, including the American No. 1,¹⁶ the Japanese, and the

TABLE IX.

Immunological Relation of the Guayaquil Mouse Leptospira to Leptospira icteroides.

Immune Serum 904, produced with Group 30 strain, injected on Dec. 20, 1918, Jan. 6, 14, 22, Feb. 3, 15, 1919.

<i>Leptospira icteroides</i> strain.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
E. Ch.	Slight agglutination without immobilization.	Doubtful.	No protection.
A. Ce.	No effect.	Negative.	" "
M. G.	" "	"	" "
A. A.	" "	"	" "

French. The guinea pigs previously inoculated with the killed cultures of the Guayaquil strains proved resistant to a subsequent infection, not only with homologous but also with the heterologous strains derived from Japanese, European, and American sources. This experiment indicates that the Guayaquil rodent strains of leptospira are identical with *Leptospira icterohæmorrhagiæ*.

SUMMARY.

By the inoculation of guinea pigs intraperitoneally with the emulsions of kidneys from wild rats and mice captured in Guayaquil, it was found that 67 per cent of the wild rats tested harbored in their kidneys a leptospira which produced in guinea pigs symptoms and lesions identical with those produced by *Leptospira icterohæmorrhagiæ* derived either from patients suffering from infectious jaundice in Japan or Europe, or from wild rats caught in New York.

¹⁶ This strain was so attenuated that some of the guinea pigs escaped a fatal infection and could not be used in further experiments.

Immune sera were prepared in rabbits by injecting different strains of the Guayaquil leptospira. These sera had a marked agglutinating and disintegrating influence upon the homologous strains, and also, but often to a less pronounced degree, upon the strains of *Leptospira icterohæmorrhagiæ* from other sources. Pfeiffer's phenomenon was also found to be positive, and protection was demonstrated against infection with virulent cultures of strains of *Leptospira icterohæmorrhagiæ*.

The same sera had no effect, or at most a very slight one, upon *Leptospira icteroides*. Guinea pigs inoculated with *icteroides* strains were not noticeably protected by the use of the immune sera prepared with the Guayaquil rat strains.

Guinea pigs inoculated with killed cultures of the Guayaquil strains of leptospira proved to be resistant to a subsequent infection with heterologous as well as homologous strains of *Leptospira icterohæmorrhagiæ*.

It is concluded, therefore, that the leptospira isolated from the kidneys of wild rats and mice in Guayaquil belongs to the group of *Leptospira icterohæmorrhagiæ*, and differs from *Leptospira icteroides* in its immunity reactions.

No positive transmission was obtained with kidney material from bats and an opossum.

INTESTINAL OBSTRUCTION.

II. A STUDY OF THE FACTORS INVOLVED IN THE PRODUCTION AND ABSORPTION OF TOXIC MATERIALS FROM THE INTESTINE.

BY LESTER R. DRAGSTEDT, CARL A. DRAGSTEDT, JOHN T. McCLINTOCK,
M.D., AND C. S. CHASE, M.D.

(From the Laboratories of Physiology and Pharmacology of the State University of Iowa, Iowa City.)

(Received for publication, May 22, 1919.)

Obstruction to the passage of food through the intestine leads to adverse symptoms and complete obstruction causes death. Acute obstruction in the upper part of the small intestine is more rapidly fatal than in the intestine lower down. The symptoms are those of a severe, rapidly developing toxemia. Toxic substances accumulate in the obstructed intestine, which when injected intravenously in animals or absorbed from the abdominal cavity produce symptoms similar to those arising after acute obstruction. It has been shown by Stone, Bernheim, and Whipple (1), and later by Hartwell and his associates (2), and Murphy and Brooks (3) that the production of isolated closed loops of the intestine, with the reestablishment of intestinal continuity around the isolated loop, produces symptoms similar to those following complete obstruction of the intestine at the same level. There is an accumulation of toxic materials in these intestinal loops similar to those in the obstructed intestine. In a previous study (4) it was demonstrated that these toxic substances can be formed in such isolated closed intestinal loops, with resultant toxemia, after all food materials and digestive secretions have been previously removed by careful washing with water or salt solution. It was also shown that the secretions of the intestinal mucosa are not toxic and do not give rise to the symptoms of acute obstruction when absorbed directly from the abdominal cavity. Davis and Stone (5) have found that these secretions are not toxic even when injected intravenously in animals. This is in agreement with the observation of Murphy and Brooks. The presence of bacteria in the lumen of the intestine or in isolated closed intestinal loops is necessary for the production of the characteristic toxic substances. In the absence of bacteria, as in isolated closed intestinal loops previously rendered sterile by prolonged drainage into the abdominal cavity, many changes, even to autolysis of the mucosa through occlusion of the blood supply, may occur and no untoward symptoms result. The toxic substances can be apparently formed by the action of bacteria on the secretions of the intestinal mucosa, or on material from desquamated mucosa cells.

Davis and Stone noted that whereas normal fresh intestinal juice was not toxic, if it was kept free from preservatives and unheated, it rapidly became so, producing the same effects on intravenous injection as the materials from closed loops. A rapid and profuse growth of bacteria in this secretion was noted.

However, the presence of bacteria plus a suitable substrate either in the lumen of the obstructed intestine or in closed intestinal loops does not in many cases produce the characteristic acute toxemia unless there is some factor present permitting the absorption of these toxic materials into the general circulation. Absorption of toxic materials from the intestine occurs both in clinical and experimental obstruction and after the formation of closed isolated intestinal loops in the great majority of cases. The present study was undertaken to determine the factors involved in this absorption of toxic materials and to secure additional evidence as to the manner of their production.

The experiments were performed on dogs, under complete ether anesthesia and with strict aseptic precautions.

Isolated Closed Intestinal Loops Previously Washed with Water and Ether.

It has been demonstrated many times by different workers that the production of closed isolated loops of the small intestines in dogs, in the great majority of cases, gives rise to a quickly developing rapidly fatal toxemia in all respects similar to the symptoms of complete acute obstruction of the intestine in the same region. These closed loops in the duodenum or upper jejunum are more rapidly fatal than similar loops in the ileum or colon. In every case there accumulates in the closed loops a toxic material, similar to the material found in the intestine in experimental obstruction in animals or in acute obstruction in man. If these isolated intestinal loops are previously washed with sterile water and ether about 50 per cent of the animals survive indefinitely and display no toxic symptoms following the operation.

The mucosa of such loops when examined at varying intervals after the operation is normal in appearance. Since it has been demonstrated that the presence of bacteria in closed intestinal loops is necessary for the production of toxic materials it would appear that the ether washing of these loops previous to closure must have either

inhibited the growth of bacteria and so prevented the formation of toxic substances, or it must have changed conditions so that toxins present were not absorbed. It is conceivable that the ether may have affected conditions in any of the following ways: (1) The specific bacterium causing the elaboration of the toxic product in the closed loop may have been removed or its growth inhibited. The bactericidal property of the ether itself may have been sufficient to destroy the bacteria or the hyperemia incited by the ether as an irritant may have been instrumental in aiding phagocytosis. (2) The substrate upon which bacteria act to produce the toxic product may have been washed out by the ether. Thus the ether-soluble lecithin, a constituent of bile and animal tissues generally, is split by putrefactive intestinal bacteria giving rise to the exceedingly toxic choline and neurine. (3) The secretory activity of the mucosa may have been sufficiently depressed to prevent the accumulation of enough fluids to distend the intestine to the point of rupture or to occlude its blood supply. The recovery of the cells from the effects of the anesthetic may have been sufficiently gradual to permit the establishment of an equilibrium between the amount of secretion into the lumen of the intestine and the amount of absorption. There is a possibility that the absorptive properties of the cells of the washed loop may have been increased through the removal of the lipid envelope of the cells by the ether. (4) The ether may have inhibited the formation of toxic substances within the cells of the mucosa of the closed loop, or produced some change in a possible harmful internal secretion of the mucosa of the intestine. (5) The ether may have decreased the absorption of substances from the intestine.

Experiments to Determine the Factors Involved in the Production of the Poisons of Obstruction.

It was found that if isolated intestinal loops were washed with water and ether previous to closure many animals survived the operation, never displayed any toxic symptoms, and that these loops when subsequently removed and examined contained great numbers of bacteria. Ether does not sterilize these intestinal loops and the efficacy of the

ether in prolonging the life of closed loop dogs does not depend primarily upon its bactericidal properties. A large number of antiseptics, such as alcohol, lysol, phenol, cresol, formaldehyde, mercuric chloride, silver nitrate, and chloroform, was used to wash the intestinal loops previous to closure. These chemicals did, in many cases, prolong the life of dogs with closed intestinal loops. Their efficacy, however, was not proportionate to their bactericidal efficiency and none was so effective as ether. In no case did they afford a complete sterilization of the intestinal loop or permanently inhibit the growth of bacteria.

The following experiments were done to determine whether the growth of bacteria and the production of the usual toxic substances in closed intestinal loops were prevented by the previous use of antiseptics. A dog which had survived the production of an isolated closed loop of the upper jejunum, previously washed with ether, was subsequently opened and the contents of the closed loop were removed with a syringe. About 50 cc. of fluid were obtained which, when injected into the abdominal cavity of another dog, caused a severe toxemia and death in 6 hours. In another animal an isolated loop of the transverse colon was made, washed with sterile water and 70 per cent alcohol, the ends were closed, and the continuity of the intestinal tract was reestablished by end to end anastomosis. This animal displayed no untoward symptoms and after 28 days was opened and the isolated loop removed. It was greatly distended, containing 225 cc. of turbid chocolate-colored fluid. The mucosa was normal. 6 cc. of this fluid were injected intravenously in another dog, and produced a profound prostration and death in 10 hours. Both the jejunal and the colon loops contained large numbers of bacteria.

These experiments established the fact that it is impossible to sterilize even a short piece of the intestinal tract by the use of chemical antiseptics. They indicate that the part played by ether is not that of a bactericide and that it does not markedly inhibit the production of toxic materials in intestinal loops. The absorption of these toxic substances is for some reason prevented. It is possible for many times the lethal dose of these poisons to remain in closed loops of the jejunum or colon without the production of toxic symptoms.

Experiments to Determine the Factors Involved in the Absorption of Poisons from the Obstructed Intestine.

The fact that closed loops of the upper jejunum in dogs are rapidly fatal, in the great majority of cases, while identical loops previously washed with ether are compatible with life in approximately 50 per cent of cases, is significant and affords an opportunity for the study of the factors involved in the absorption of the poisons of obstruction. Washing with ether does not prevent the formation of toxic materials in closed intestinal loops but it does prevent the absorption of these materials in many instances. If closed loops of the upper jejunum, untreated with ether, are made, the animal usually dies in from 1 to 5 days with symptoms of an acute toxemia, appearing only from 10 to 24 hours before death. At autopsy there is usually a perforation of the loop and a general peritonitis. Many animals, however, die and the autopsy discloses an intact loop, in most cases enormously distended, discolored, with a necrotic mucosa, but no peritonitis. It is noteworthy that these closed loop dogs display no adverse symptoms until shortly before they die at a time when apparently the accumulation of secretions in the loop has caused sufficient distention to occlude the blood supply to the mucosa. It seemed possible that ether may have prevented the onset of toxemia in closed loop dogs by preventing this distention of the loop.

Ether acts as a local narcotic causing a coagulation of the cell protoplasm. This coagulation might at least temporarily depress the secretion of intestinal juice. If this is the important factor then other substances with strong astringent but without bactericidal properties should be equally effective.

Isolated loops of the upper jejunum were made in eight dogs, flushed with water and subsequently with 8 per cent alum (aluminum potassium sulfate), the ends infolded and closed, and the continuity of the intestinal tract was reestablished by end to end suture. Two of these dogs survived the operation and remained in good health 6 months later. Six of the dogs died within the first 5 days with a perforation of the loop and a general peritonitis. Similar isolated jejunal loops were made in fourteen dogs, but a 6 per cent solution of tannic acid was used to flush the loop instead of alum. Of these dogs four con-

tinued in perfect health for months after the operation; the remainder died after periods varying from 6 to 20 days.

Apparently, simple astringents with no germicidal properties are just as effective in preventing the symptoms of toxemia in dogs with closed intestinal loops as are the antiseptics. It was evidently the astringent properties of the ether and the other chemicals rather than their bactericidal properties that account for the results produced.

The factor of distention in short closed intestinal loops is of paramount importance in the production of the toxemia, since if the distention is prevented in the majority of cases toxemia does not occur.

It has been emphasized by Hartwell and his associates and by Murphy and Brooks as well as by ourselves that toxic materials are not rapidly absorbed through the normal mucosa. This fact is well illustrated in the following experiments. Three dogs, which had survived the production of closed ether-washed loops of the upper jejunum for several weeks, were subsequently reopened. Into the closed loop of one dog the contents of a normal intestine with its bacterial flora, into the loop of the second the contents of an obstructed intestine, and into the loop of the third the evaporated ether washings of an isolated jejunal loop, containing a possible bacterial substrate, were injected. None of the animals displayed any unusual symptoms after recovering from the laparotomy and continued in good health. It has been noted before that dogs may survive the production of closed loops of the jejunum and may continue in perfect health although the loop may contain many times a lethal dose of toxic substances. This we believe is due to a protective function on the part of the intestinal mucosa which is able to inhibit or diminish the systemic effect of poisonous substances found in the alimentary tract. It is dependent upon the capacity of the mucosa cells for physiological selection by which these cells, while they absorb certain substances (in apparent defiance of physical laws) that diffuse with difficulty, do not, on the other hand, permit others to pass which may be more diffusible. This protective capacity, however, has a limit, or intoxication from the alimentary tract could not occur. Indeed it has been our experience that if long (1 to 6 feet) closed loops of the intestine, previously treated with ether, are made, no symptoms may be apparent for 2 weeks, but shortly thereafter a slowly developing toxemia may ensue and death

occur in the course of 5 or 6 days. The mucosa of these loops may be perfectly normal to both gross and microscopic examination. With short loops any poisons which may have found their way through the mucosa were apparently removed from the circulation or detoxified by the liver. With the long loops, however, the liver was not able to cope with the large quantity of toxic substances and toxemia resulted.

The following experiment emphasizes the importance of the protective action of the normal intestinal mucosa. An isolated loop (12 cm. in length) was made in the jejunum, washed with water and ether, both ends were closed, and the continuity of the alimentary tract was reestablished by end to end anastomosis around the loop. The animal quickly recovered and displayed no subsequent toxic symptoms. 1 month later a second laparotomy was done, the isolated closed loop filled with a 25 per cent solution of magnesium sulfate, dropped back, and the abdomen closed. 3 days later the animal died with the usual symptoms and autopsy revealed a perforated loop with a general peritonitis. It was demonstrated before that ether washing of the loop previous to closure does not prevent the formation of toxic materials. It is well known that a strong solution of magnesium sulfate will attract fluids into the lumen of the intestine acting through the alteration of osmotic pressure conditions. Evidently the distention of the closed loop produced by the attraction of liquids was the immediate cause of the toxemia and death. The animal was able to survive the production of a closed jejunal loop which contained more than a lethal dose of toxic materials without symptoms but died when this loop was distended. The conclusion seems inevitable that the injury to the mucosa produced by the distention has deprived these cells of their protective function and permitted an overwhelming amount of toxic material to enter the blood stream. Evidence obtained from the other workers in this field as well as from our own experiments indicates that the distention of the intestine injures the mucosa by occluding its blood supply. Once the protective layer of intestinal epithelium has been functionally destroyed absorption takes place as from the peritoneal cavity whose serosa exerts no selective action.

It is not probable that simply an irritation of the mucosa by retained substances in the obstructed intestine is the factor which damages the cells, destroys their power of selective absorption, and permits the absorption of the toxins of obstruction. There is no evidence that the toxins of obstruction are qualitatively different from the toxic materials present in the normal digestive tract and if simply an irritation of the mucosa were the important factor in their absorption a pronounced toxemia should occur in any enteritis.

Experiments to Determine the Possibility of a Non-Bacterial Origin of the Poisons of Obstruction.

In view of the fact that some of the workers, who have done a great deal of experimental work on the problem of intestinal obstruction, hold to the view that the poisons responsible for the toxemia arise independently of the intestinal bacteria, it is necessary to detail the experiments which have forced us to reject these theories. It has been postulated by a number of workers and considerable experimental and clinical evidence has been adduced to show that the toxemia incident to obstruction of the intestinal tract is due to the absorption of poisons formed in the intestinal mucosa. These poisons may be either normal products of the lining cells which, because of the obstruction, are abnormally absorbed or they may be the results of an abnormal activity on the part of the intestinal mucosa induced by the condition of obstruction. Thus Draper (6) holds that the toxemia in intestinal obstruction is due to an aberrant activity of the duodenal and probably the pancreatic cells. Whipple, Stone, and Bernheim (7) believe that death in these cases of obstruction is due to the absorption directly into the blood stream of a perverted secretion of the duodenal or upper jejunal mucosa. They assume a disturbed physiological balance of the mucosa by which abnormal products are formed and secreted into the blood stream. This toxic secretion is a proteose and, it is stated, can be formed in a mucosa in which there is no gross evidence of disturbance. Thus both of these theories exclude the intestinal bacteria as an important factor in the production of the toxic materials. A number of experiments were done to obtain evidence on this point and in no case do we believe

that the experimental evidence warrants the assumption of a non-bacterial origin of the poisons concerned in obstruction.

In our first study (4) the following points were brought out: (a) Open isolated loops of the duodenum, the jejunum, or the ileum do not give rise to a toxemia when their secretions are drained directly into the abdominal cavity. South and Hardt (8) had independently found that animals could survive the production of open isolated loops of the small intestine, and this has been abundantly confirmed. (b) Closed isolated intestinal loops previously washed with ether may produce no symptoms in 50 per cent of cases. (c) Closed isolated intestinal loops previously rendered sterile by prolonged drainage into the abdominal cavity produce no untoward symptoms. (d) Sterile closed intestinal loops produce no adverse symptoms even on complete autolysis of the mucosa through occlusion of the blood supply. (e) An ether-washed closed intestinal loop (not sterile), which had caused no symptoms, produced the typical toxemia and death on occlusion of its blood supply.

A consideration of these experiments indicates the improbability of a toxic secretion of the mucosa cells. It has been amply demonstrated that the normal secretions of these cells are not toxic when absorbed from the abdominal cavity or injected intravenously. The production of closed intestinal loops does not always cause toxic symptoms if the factors of bacterial growth and distention of the loops are controlled. If the toxemia were dependent upon toxic secretions of mucosa cells it should uniformly occur when closed loops are made, and furthermore one would expect a slowly developing toxemia from the time of production of the loop instead of a fulminant toxemia coincident with distention of the loop and occlusion of its blood supply. It might be conceived that distention of the intestinal loop is a necessary stimulus to the mucosa cell to produce its toxic product. But if bacteria have been previously excluded from such a closed loop it may distend to the point of rupture, or its blood supply be occluded by ligation and no toxic symptoms result. However, if bacteria are present, either of these procedures will cause typical symptoms and death. It might then be conceived that bacteria are a necessary stimulus to the production of a toxic secretion by the mucosa. However, if bacteria are present in a closed loop but the distention of the loop is prevented by substances which inhibit intestinal secretion, no toxic symptoms result. It would be too far fetched to assume both

the presence of bacteria plus a distention of the intestinal wall as a stimulus to dying cells to produce a specific toxic secretion. We know that intestinal bacteria acting upon proteins or their split products can elaborate poisons which produce symptoms identical with those of obstruction, and all the evidence we have obtained points to these substances as the important constituents of the toxins of obstruction.

It might be conceived also that obstruction and accompanying injury to the intestinal mucosa may have thrown out of function an organ essential to life. Several workers have stated, from experimental evidence, that the mucosa of the upper duodenum has a specific vital function, aside from the secretion of succus entericus and the manufacture of secretin, and that disturbance of this function rapidly causes death.

S. A. Matthews (9) reasons, from experiments in which the entire duodenum in dogs was resected, that the duodenal mucosa is as necessary for life as the adrenals or parathyroids probably through some hormone function other than that concerned with the elaboration of secretion. A. P. Matthews (10) states that if experiments are made so that the duodenal juice (succus entericus) is drained to the exterior through a fistula, the animals die with apparently the symptoms of complete extirpation of the duodenum. He suggests that death may be due to the rapid excretion of some necessary substance through the duodenum to the exterior or to the loss of some substance normally elaborated by the duodenum which is necessary to the function of the intestine lower down.

It has long been known that resection of varying lengths of the jejunum, ileum, or colon, and even a complete removal of the stomach may produce no symptoms other than can be accounted for as a result of the loss of a digestive and absorptive organ. It has also been quite generally recognized that surgical interference with the duodenum is an extremely dangerous procedure and that excision of even parts of the duodenum is usually fatal. The symptoms produced in dogs after complete extirpation of the duodenum, as described by S. A. Matthews, and those produced by drainage of duodenal juices, as described by A. P. Matthews, were so much like those produced by simple occlusion of the upper intestine, that it was considered advisable to repeat their experiments. A complete account of our work has been published in another paper (11) and accordingly only a brief summary need be given here. It was found that removal of varying lengths of the jejunum and ileum produced no other effect than some nutritional disturbance, attributable to the loss of an important digestive and absorptive organ. The mucosa of the combined jejunum and ileum does not secrete or manufacture a necessary substance and animals can survive for months after the removal of the small intestine, the duodenum and colon remaining intact. A dog was kept living 3 months after a complete removal of the pyloric

part of the stomach, the entire duodenum, and the upper jejunum. This was confirmed by Grey (12), who was able to keep a dog 9½ months after complete removal of the duodenum.

Moorhead and Landes,¹ state that they have a dog in good health and gaining weight 3 months after the removal of the entire duodenum. Thus the mucosa of the alimentary tract does not possess a function comparable with the adrenals and parathyroids. Dogs were kept living 10 and 12 days after the establishment of a fistula which drained the entire duodenum. There is no evidence that the duodenum excretes in the succus entericus any substance necessary for life, or for the function of the intestine lower down.

DISCUSSION.

It has been definitely determined that death resulting from acute obstruction of the intestine is due to a toxemia and that the responsible toxic substances are formed in the obstructed intestine. These toxic substances can be formed even if all food materials, end-products of digestion, and the secretions of the stomach, liver, and pancreas have been carefully excluded. The secretion of the intestinal mucosa is not toxic either when absorbed from the abdominal cavity or injected intravenously. The mucosa of the alimentary tract (stomach, duodenum, jejunum, ileum, or colon) does not elaborate an internal secretion which is necessary to life, or which could be disturbed by the conditions of acute obstruction so as to account for the symptom complex of that condition. The presence of bacteria in the lumen of the intestine is necessary for the production of the characteristic toxic substances and in their absence these substances do not form. They are produced by the action of the intestinal bacteria on proteins or their split products. In the absence of food, gastric juice, bile, or pancreatic juice, these bacteria can produce the characteristic toxic substances from the intestinal juice or from the proteins of desquamated mucosa cells. The important poisons will not provoke the appearance of immune bodies when injected in experimental animals (13) and we were not able to demonstrate that an animal can become immune to the toxemia of acute obstruction (14). Toxic amines are produced

¹ Moorhead, J. J., and Landes, H. E., personal communication.

by the action of various intestinal bacteria on amino-acids and the evidence more and more points to these substances as the important agents in the toxemia of acute intestinal obstruction.

The toxic substances arising in the lumen of the obstructed intestine are not readily absorbed through a normal mucosa, a point emphasized by Hartwell and his associates and by Murphy and Brooks. Nor are they absorbed to any great extent through the mucosa of a closed intestinal loop until this mucosa has been injured by the distention of the loop and the consequent interference with the blood supply. If this distention is prevented by any means absorption of poisons in quantities greater than can be cared for by the liver and other tissues does not occur. Thus it appears that the injury to the mucosa cells, either as a result of the sudden distention brought about by conditions of obstruction or by any other factors which interfere with the blood supply to the mucosa (strangulation, etc.), is an important factor in the absorption of toxic substances from the intestine. There can be no doubt that necrosis of the mucosa greatly facilitates the absorption of intestinal poisons; but it is incorrect to say that intestinal poisons, *i.e.* those found in obstruction, cannot be absorbed through a normal mucosa. The protective action of the intestinal mucosa exercised through its properties of selective absorption is not absolute, but that it is of great significance is shown by the fact that an animal can take care of the amount of poisons absorbed through the normal mucosa of a short closed intestinal loop, which has been treated with astringents, but that as soon as this mucosa becomes necrotic an overwhelming amount of toxic materials gains entrance to the blood stream, with toxemia and death occurs. The absorption in these cases cannot be different from absorption from the peritoneal cavity.

CONCLUSIONS.

1. It is impossible to sterilize the intestine by the use of chemical antiseptics even when these are applied directly to the mucosa of isolated segments.

2. The mucosa of the alimentary tract does not elaborate an internal secretion which is necessary to life, or a secretion which could be disturbed by the conditions of acute obstruction so as to account for the symptom complex of that condition.

3. The substances responsible for the toxemia in acute obstruction are produced by the action of intestinal bacteria on proteins or their split products.

4. An injury to the intestinal mucosa, particularly that resulting from disturbances of the blood supply to the intestine, greatly facilitates the absorption of these poisons. The work of Hartwell and his associates and that of Murphy and Brooks on this point are confirmed.

BIBLIOGRAPHY.

1. Stone, H. B., Bernheim, B. M., and Whipple, G. H., *Bull. Johns Hopkins Hosp.*, 1912, xxiii, 159.
2. Hartwell, J. A., and Hoguet, J. P., *Am. J. Med. Sc.*, 1912, cxliii, 357; *J. Am. Med. Assn.*, 1912, lix, 82. Hartwell, J. A., *J. Exp. Med.*, 1913, xviii, 139. Hartwell, J. A., Hoguet, J. P., and Beekman, F., *Arch. Int. Med.*, 1914, xiii, 701.
3. Murphy, F. T., and Brooks, B., *Arch. Int. Med.*, 1915, xv, 392.
4. Dragstedt, L. R., Moorhead, J. J., and Burcky, F. W., *J. Exp. Med.*, 1917, xxv, 421.
5. Davis, D. M., and Stone, H. B., *J. Exp. Med.*, 1917, xxvi, 686.
6. Draper, J. W., *J. Am. Med. Assn.*, 1916, lxvii, 1080; 1911, lvii, 1338.
7. Whipple, G. H., *J. Exp. Med.*, 1912, xv, 259. Whipple, G. H., Stone, H. B., and Bernheim, B. M., *ibid.*, 1913, xvii, 286, 307; 1914, xix, 144, 166.
8. South, F. L., and Hardt, L. L. J., *Arch. Int. Med.*, 1918, xxi, 292.
9. Matthews, S. A., *J. Am. Med. Assn.*, 1910, lv, 293.
10. Matthews, A. P., *Physiological chemistry*, New York, 1915, 388.
11. Dragstedt, L. R., Dragstedt, C. A., McClintock, J. T., and Chase, C. S. *Am. J. Physiol.*, 1918, xlvi, 584.
12. Grey, E. C., *Surg., Gynec. and Obst.*, 1919, xxviii, 36.
13. Dragstedt, C. A., Dragstedt, L. R., and Chase, C. S., *Am. J. Physiol.*, 1918, xlvi, 366.
14. Dragstedt, C. A., and Moorhead, J. J., *J. Exp. Med.*, 1918, xxvii, 359.

A STUDY OF PNEUMOCOCCI REACTING WITH ANTIPNEUMOCOCCUS SERA OF TYPES I, II, AND III, WITH AN OBSERVATION OF A MUTATION OF ONE OF THE STRAINS.*

By MILDRED C. CLOUGH, M.D.

(From the Medical Clinic of the Johns Hopkins Hospital, Baltimore.)

(Received for publication, April 29, 1919.)

In the routine study of the pneumococci isolated from cases of lobar pneumonia and other infections in the wards of the Johns Hopkins Hospital from 1915-18, certain strains were encountered which were agglutinated almost equally well by antipneumococcus sera of Types I, II, and III.¹ In making these tests progressively increasing dilutions of serum were used from 1:2 to 1:64. As a rule, agglutination did not occur in such high dilutions of the sera as in the case of typical members of the fixed types in the homologous serum, nor was agglutination so prompt in appearing. No agglutination of these strains occurred in normal horse serum on isolation.

The existence of such strains has been noted by others. Mathers (1) in 1915 described such agglutination in 16 out of 31 pneumococci, classified as Group IV, isolated from patients with lobar pneumonia. This agglutination was usually absent in the higher dilutions of serum, but with two of the strains agglutination occurred with Sera I and II in a dilution of 1:128.

Wollstein and Benson (2) isolated one such strain from the sputum of a child with lobar pneumonia. The morphology of the organism was that of a pneumococcus, but inulin was not fermented, and it was "insoluble in bile and in optochin." It was, therefore, considered to be a streptococcus.

Blake (3) in a recent article states that a small number of these strains has been isolated at the Hospital of The Rockefeller Institute. He classified them as ordinary Group IV pneumococci, and considered the agglutination a non-

* This work was done in part under the Mary Putnam Jacobi Fellowship of the Women's Medical Association of New York City.

¹ These sera were obtained from the Hospital of The Rockefeller Institute, through the kindness of Dr. Rufus Cole.

specific reaction, such as may occur with other bacterial groups when undiluted serum of high agglutinin titer is used. He showed that by the use of the more delicate precipitin test a limited zone of non-specific reaction exists with pneumococci of all four types.

Stryker (4) produced strains agglutinating in antipneumococcus sera of both types (I and II) by growing Type I and II pneumococci in their homologous immune sera. Coincident with the change in agglutinability there was a decrease in virulence, with inhibition of capsule formation, and increase of phagocytability in normal serum. Absorption of Serum I or II with strains grown in homologous immune serum removed all the agglutinins for homologous and heterologous immune serum-grown strains, but not for homologous normal serum-grown strains. Saturation of serum with homologous normal serum-grown strains removed all the agglutinins for them, and also for heterologous immune serum-grown strains. After passage through a few animals these strains reverted to their original types.

Methods.

The strains reported here were morphologically typical pneumococci. Their cultural reactions will be discussed in detail later. The serological reactions of these pneumococci were studied as far as possible by the following methods.

The macroscopic agglutination of the strains was tested in progressively increasing dilutions (from 1:2 to 1:64) of Antipneumococcus Sera I, II, and III, and of normal horse serum, by adding to them an equal volume (0.25 cc.) of a 24 hour broth culture of the strain in question. These tests were incubated 1 hour in the 37°C. water bath, read, and allowed to stand in the ice chest over night, and read again the next day. The figures in the tables indicate the highest dilution of serum in which definite macroscopic agglutination was visible, without the use of a lens. Some of the strains became slightly agglutinable in normal horse serum after prolonged cultivation on blood agar, but the titer of agglutination in normal horse serum was always lower than in the antipneumococcus sera. Animal passage for a time removed this tendency to agglutination in normal horse serum.

Phagocytosis of these atypically agglutinable strains in the antipneumococcus sera was tested. Equal volumes of bacterial suspension, serum, and a suspension of washed human leucocytes were mixed in capillary pipettes, and incubated at 37°C., as in Wright's

method. Films of the mixture were then made, and stained with Hastings' polychrome methylene blue stain. The technique of preparing these suspensions, and the precautions to be observed have been described by Clough (5). In all the tests control preparations were made with normal horse serum, and the time of incubation was so adjusted as to give a maximum degree of phagocytosis in the tests with no phagocytosis in the controls. In these mixtures the presence or absence of microscopic agglutination was also noted. The degree of phagocytosis and microscopic agglutination present is recorded as (+) slight but definite; (+²) marked; and (+³) maximal.

Tests were then made to determine whether or not these atypically agglutinable strains elaborated a precipitable substance reacting with all three types of antipneumococcus serum, such as Dochez and Avery (6) have shown in the case of the fixed types with their homologous serum. The peritoneum of mice infected with these organisms was washed out with several cubic centimeters of salt solution, and this fluid was centrifugalized until clear. The clear supernatant fluid was then layered over sera of the various types. After noting the presence or absence of a ring at the junction of the two fluids, the tubes were shaken, and any diffuse clouding or flocculation was noted. This technique was suggested by Blake (3) as a quick method of type determination. The degree of the reactions is indicated roughly by the signs +, +², +³, as in the phagocytic tests.

Agglutinin absorption tests were carried out as follows: The strains used for the absorptions were grown in 150 cc. flasks of meat infusion broth for 24 hours, and then centrifugalized at high speed until all the organisms were thrown down. The broth was pipetted off, and the organisms were resuspended in salt solution, and again centrifugalized. After removing the supernatant salt solution the organisms were killed by heating for 20 minutes in a water bath at 56°C. They were then emulsified in 5 cc. of the serum to be tested, and the mixtures incubated 2 hours in the thermostat, and left over night in the ice box. After centrifugalization the serum was tested by macroscopic agglutination tests with the strain used for the digestion to see whether absorption was complete. This process was repeated until the serum was exhausted. Usually three such absorptions were required.

The protective power of the antipneumococcus sera was tested with two strains (L and N). These organisms could be rendered sufficiently virulent by animal passage to kill mice in a dose of 0.000001 cc. In making these tests 24 hour meat infusion broth (titrated to pH 7.8) cultures were used in doses of 0.1 up to 0.000001 cc. 0.2 cc. of each culture dilution was mixed with an equal volume of the serum to be tested, and the mixture injected intraperitoneally into mice. The protective power of sera of Types I, II, and III was tested in this way, with a series of culture dilutions with normal serum as a control. With one strain the protective power of an homologous immune serum was also tested. In these tests the time of death of the inoculated mice was noted, and the presence or absence of pneumococci in the peritoneum and heart's blood was determined by films or cultures or by both. The inoculated mice were watched for 10 days. Animals dying in less than 10 days were considered to be protected if films and cultures from the heart and peritoneum showed no organisms.

With the other seven of these strains it was impossible to obtain satisfactory protection tests, because their virulence could not be increased sufficiently. With two strains (V and R) passage through ten mice, and with another strain (T) through fourteen mice in rapid succession did not result in sufficient increase in virulence to kill mice in doses of 0.1 cc. of a 24 hour broth culture.

Immune sera were prepared to seven of the nine pneumococci studied, by intravenous injection of rabbits, first with killed cultures, and later by increasing doses of broth cultures of the living organisms. These injections were continued at intervals of 2 days until the serum agglutinated the homologous organisms in a dilution of at least 1:250. Each strain was then tested in all the sera by means of phagocytic and agglutinative reactions.

The study of these strains was attended with great technical difficulty, and not all the tests could be applied to each strain. Protection tests were possible in only two instances on account of the low virulence of the rest of the strains. Several of them required frequent animal passage in order to keep them non-agglutinable and non-phagocytatable in normal serum, and three eventually became entirely avirulent for animals and had to be discarded.

Description of the Strains.

Strain 1.—V. Acute lobar pneumonia. Isolated March, 1915. *Source.*—Lung puncture. *Morphology.*—Gram-positive, lance-shaped diplococcus with small capsules. *Blood agar.*—Colonies green, small, discrete, and rather dry. *Broth.*—Diffusely clouded. *Litmus milk.*—Acidified and coagulated. *Bile.*—Partially soluble. *Inulin.*—Not fermented. *Glucose ascitic fluid agar.*—Precipitated. *Virulence.*—Low. The growth from a blood agar slant frequently failed to kill a mouse, and after ten successive animal passages 0.1 cc. of a broth culture failed to kill.

Serological Tests.

Serum.	Macroscopic agglutination.		Phagocytosis.	Microscopic agglutination.
	1 hr.	24 hrs.		
Type I.....	1: 16	1: 64	+ ³	+ ³
" II.....	1: 16	1: 64	+ ³	+ ³
" III.....	1: 16	1: 16	+ ³	+ ³
Normal horse.....	0	1: 8	0	0

Strain 2.—T. Bronchopneumonia with multiple abscesses and double empyemas. Isolated October, 1915. *Source.*—Sputum culture, and culture of lung at autopsy. *Morphology.*—Gram-positive, lance-shaped diplococcus occurring in short chains at first, and longer chains after prolonged cultivation. Small capsules. *Blood agar.*—Colonies yellowish green, rather small, discrete, fairly moist. *Broth.*—Diffuse turbidity at first, later slightly granular. *Bile.*—Partially soluble. *Inulin serum water.*—Acidified and coagulated. *Glucose ascitic fluid agar.*—Not precipitated. *Virulence.*—Low. The growth of a blood agar slant occasionally failed to kill a mouse, and after fourteen passages through mice in rapid succession 0.1 cc. of a broth culture failed to kill.

Serological Tests.

Serum.	Macroscopic agglutination.		Phagocytosis.	Microscopic agglutination.
	1 hr.	24 hrs.		
Type I.....	1: 8	1: 8	+ ²	+ ³
" II.....	1: 32	1: 32	+ ²	+ ³
" III.....	1: 4	1: 16	+ ²	+ ³
Normal horse.....	0	1: 2	0	0

Agglutinin absorption tests.—Specimens of Type I and II serum were exhausted with the homologous pneumococci, and other specimens of these sera with Strain T, by the method already described. Exhaustion of both these sera with Strain T did not reduce their activity towards the homologous (Type I or II) organisms, and in general reduced but little, if at all, their activity toward the other strains in this series. On the other hand, exhaustion of Sera I and II with the homologous organism (Types I and II), reduced or eliminated their activity towards all the strains which could still be satisfactorily tested. Since by the time these sera were obtained some of the strains had become agglutinable in normal horse serum, clear-cut results could not be obtained with them.

Strain 3.—R. Bronchiectasis. Isolated May, 1915. *Source.*—Sputum (mixed culture). *Morphology.*—Gram-positive, lance-shaped diplococcus with small capsules. *Blood agar.*—Colonies resembled those of previous strain. *Broth.*—Diffuse turbidity. *Litmus milk.*—Acidified and coagulated. *Bile.*—Soluble. *Inulin serum water.*—Acidified and coagulated. *Glucose ascitic fluid agar.*—Not precipitated. *Virulence.*—Low. The growth from a blood agar slant usually killed mice, but not uniformly.

Serological Tests.

Serum.	Macroscopic agglutination. 24 hrs.	Phagocytosis.	Microscopic agglutination.
Type I.....	1: 16	+ ²	+ ³
" II.....	1: 32	+ ²	+ ³
" III.....		+ ²	+ ³
Normal horse.....	1: 2	0	0

Strain 4.—X. Normal mouth. Isolated April, 1917. This culture was lost before a thorough study of it could be made. *Source.*—Mouse inoculation with saliva. *Morphology.*—Gram-positive, lance-shaped diplococcus with moderate sized capsules. *Blood agar.*—Colonies resembled those of previous strain. *Broth.*—Diffuse turbidity. *Inulin.*—Fermented.

Serological Tests.

Serum.	Macroscopic agglutination.		Phagocytosis.	Microscopic agglutination.
	1 hr.	48 hrs.		
Type I.....	1: 4	1: 32	+	+ ²
" II.....	1: 8	1: 64	+ ²	+ ³
Normal horse.....	0	1: 2	0	0

Strain 5.—Ba. Acute lobar pneumonia; chronic alcoholism; diabetes; diabetic coma. Isolated April, 1916. *Source.*—Sputum culture, lung puncture post mortem, and culture from heart's blood immediately post mortem,—four colonies per cc. This strain is of the greatest interest on account of certain changes in its cultural characteristics and immunological reactions which occurred while it was under observation. When first isolated this organism reacted like an ordinary atypical Type II pneumococcus. These reactions and the changes which occurred in the strain will be discussed later. After 8 months cultivation on blood agar this strain was found to be agglutinated by all three antipneumococcus sera. For convenience this form of the strain will be called Ba. *Morphology.*—Gram-positive, small, lance-shaped diplococcus with small capsules. *Blood agar.*—Colonies moderate sized, yellowish green with slight intensification of color at the edge of the colony, and a narrow zone of slight clearing around the colony. On a blood agar slant an intense green discoloration of the medium was produced around the water of condensation. *Broth.*—Diffuse turbidity. *Bile.*—Partially soluble. *Inulin serum water.*—Not fermented. *Glucose ascitic fluid agar.*—Precipitated. *Virulence.*—Low. The growth from a blood agar slant often failed to kill a mouse.

Serological Tests.

Serum.	Macroscopic agglutination. 24 hours.	Phagocytosis.	Microscopic agglutination.	Precipitin tests.
Type I.....	1:32	+ ³	+ ³	+ ²
“ II.....	1:64	+ ³	+ ³	+ ²
“ III.....	1:4	+ ³	+ ³	+ ²
Normal horse.....	0	0	0	0

The second group of strains could be studied more thoroughly on account of their relatively higher virulence, and because they remained resistant to phagocytosis and agglutination in normal serum after cultivation.

Strain 6.—H. Encapsulated abscess in the region of the appendix; signs of an old peritonitis at operation. Isolated January, 1916. *Source.*—Culture of abscess at operation. *Morphology.*—Gram-positive, lance-shaped diplococcus with an occasional short chain. Moderate sized capsules. *Blood agar.*—Colonies yellowish green, discrete, fairly moist. *Broth.*—Diffuse turbidity. *Litmus milk.*—Acidified. *Bile.*—Soluble. *Inulin serum water.*—Not fermented when tested 15 months after isolation, but 4 months later, after passage through four mice, the strain had acquired or regained the ability to ferment inulin. *Glucose ascitic fluid agar.*—Not precipitated. *Virulence.*—0.1 cc. of a broth culture killed a mouse after eight successive mouse passages.

Serological Tests.

Serum.	Macroscopic agglutination.			Phagocytosis.	Microscopic agglutination.	Precipitin tests.
	1 hr.	24 hrs.	48 hrs.			
Type I.....	0	1: 32	1: 64	+	+ ²	+
" II.....	0	1: 16	1: 64	+ ²	+ ²	+ ²
" III.....	1: 4	1: 64	1: 64	+ ²	+	+
Normal horse.....	0	0	0	0	0	0

Strain 7.—F. Bronchopneumonia following diphtheria. Isolated July, 1917. *Source.*—Culture from lung at autopsy. *Morphology.*—Gram-positive, lance-shaped diplococcus with moderate sized capsules. *Blood agar.*—Colonies green, discrete, moist. *Broth.*—Diffuse turbidity. *Bile.*—Soluble. *Inulin serum water.*—Acidified and coagulated. *Glucose ascitic fluid agar.*—Not precipitated. *Virulence.*—0.001 cc. of a broth culture killed a mouse once, but not regularly. The virulence could not be raised above this even after twelve animal passages.

Serological Tests.

Serum.	Macroscopic agglutination.			Phagocytosis.	Microscopic agglutination.	Precipitin tests.
	1 hr.	24 hrs.	48 hrs.			
Type I.....	1: 4	1: 16	1: 64	+ ³	+ ³	+ ²
" II.....	0	1: 8	1: 64	+ ³	+ ²	+ ²
" III.....	1: 8	1: 32	1: 64	+ ²	+	+
Normal horse.....	0	0	0	0	0	0

Strain 8.—L.² Pneumococcus meningitis. Isolated March, 1918. *Source.*—Blood culture and spinal fluid culture. *Morphology.*—Gram-positive, lance-shaped diplococcus with moderate sized capsules. *Blood agar.*—Colonies moderate sized, yellowish green with slightly lighter centers and darker edges, and a very slight zone of clearing around the colony. *Broth.*—Diffuse turbidity. *Bile.*—Soluble. *Inulin serum water.*—Acidified and coagulated. *Glucose ascitic fluid agar.*—Not precipitated. *Virulence.*—High. After cultivation for a year on blood agar and passage through two mice, 0.000001 cc. of a 24 hour broth culture still suffices to kill a mouse within 2 days.

² This strain was obtained through the kindness of Dr. T. P. Sprunt.

Serological Tests.

Serum.	Macroscopic agglutination.			Phagocytosis.	Microscopic agglutination.	Precipitin tests.
	2 hrs.	24 hrs.	48 hrs.			
Type I.....	1:4	1:64	1:64	+	+	+ ²
“ II.....	1:4	1:16	1:64	+	+	+ ²
“ III.....	1:8	1:16	1:16	+	+	+
Normal horse.....	0	0	1:2	0	0	0

Protection Tests.

Mouse No.	Dose of culture.	+Normal serum.	+Type I serum.	+Type II serum.	+Type III serum.
	cc.	hrs.	hrs.	hrs.	hrs.
1	0.1	Not done.	22	22	18
2	0.01	“ “	48	26	42
3	0.001	22	S.	S.	S.
4	0.0001	24	“	“	“
5	0.00001	120	“	“	“
6	0.000001	48	“	“	“

The figures denote the number of hours between inoculation and death. Cultures or films showed pneumococci in the heart's blood and peritoneum in all cases. S. indicates that the mouse survived 10 days or longer, or died accidentally with negative cultures from the heart's blood and peritoneum. (Nos. 3 and 5 in the series protected with Type I serum.)

This test was repeated on another occasion with sera of Types I and II, and practically the same results were obtained. In this series all the mice protected with Type I serum, except Nos. 1 and 2, survived the full 10 day period, while all the control mice died with positive films and cultures.

Strain 9.—N. Acute lobar pneumonia. Isolated February, 1917. *Source.*—Sputum culture. *Morphology.*—Gram-positive, lance-shaped diplococcus with moderate sized capsules. *Blood agar.*—Colonies moderate sized, yellowish green, discrete, moist. *Broth.*—Diffuse turbidity. *Litmus milk.*—Acidified. *Bile.*—Soluble. *Inulin serum water.*—Acidified and coagulated. *Glucose ascitic fluid agar.*—No precipitation. *Virulence.*—High. 0.000001 cc. of a 24 hour broth culture sufficed to kill a mouse.

Serological Tests.

Serum.	Macroscopic agglutination.			Phagocytosis.	Microscopic agglutination.	Precipitin tests.
	1 hr.	24 hrs.	48 hrs.			
Type I.....	1:2	1:32	1:64	+ ³	+ ³	+ ²
" II.....	1:4	1:32	1:64	+ ³	+ ³	+ ²
" III.....	1:2	1:64	1:64	+ ²	+	+
Normal horse.....	0	0	0	0	0	0

Protection Tests.

Mouse No.	Dose of culture.	+Normal serum.	+Type I serum.	+Type II serum.	+N immune serum.
	cc.	hrs.	hrs.	hrs.	
1	0.5	Not done.	Not done.	Not done.	S.
2	0.1	" "	24	19	"
3	0.01	24	S.	S.	"
4	0.001	40	"	"	"
5	0.0001	60	"	"	"
6	0.00001	84	"	"	"
7	0.000001	36	"	"	"

Films or cultures showed pneumococci in the peritoneum and heart's blood in all cases. No. 3 with Type I serum, and No. 3 with N immune serum died accidentally with negative cultures from the peritoneum and heart's blood. This test was repeated with practically identical results.

Agglutinin absorption tests.—Type I serum was absorbed repeatedly with a typical Type I strain, and another specimen of Serum I with Strain N; and Type II serum with a typical Type II strain, and another specimen with Strain N, until completely exhausted, as described previously. These sera were then tested with typical Type I and II strains, and with the last four strains just described. The results obtained are given in Tables I and II.

These tables show that absorption of serum of Type I or II with the homologous organism removed the agglutinins for all the strains tested. On the other hand, absorption of these sera with Strain N removed the agglutinins for that strain only, and not for the homologous Type I or II strain, or for Strains H, F, and L. The removal of the agglutinins by absorption is paralleled closely by the removal of the substances stimulating phagocytosis.

TABLE I.
Macroscopic Agglutination.

Strains.	Sera.					
	Type I.	Type I, absorbed with Type I strain.	Type I, absorbed with Strain N.	Type II.	Type II, absorbed with Type II strain.	Type II, absorbed with Strain N.
Type I strain.....	1: 256	0	1: 256			
" II "				1: 128	0	1: 128
Strain N.....	1: 32	0	0	1: 32	0	0
" H.....	1: 64	0	1: 64	1: 32	0	1: 64
" F.....	1: 64	0	1: 64	1: 64	0	1: 64
" L.....	1: 32	0	1: 32	1: 8	0	1: 8

TABLE II.
Phagocytosis and Microscopic Agglutination.

Strains.	Sera.					
	Type I.	Type I, absorbed with Type I strain.	Type I, absorbed with Strain N.	Type II.	Type II, absorbed with Type II strain.	Type II, absorbed with Strain N.
Type I strain.....	+ ³ + ³	0 0	+ ³ + ³			
" II "				+ ³ + ³	0 0	+ ³ + ³
Strain N.....	+ ² +	0 0	0 0	+ ² + ³	0 0	0 0
" H.....	+ +	0 0	+ +	+ +	0 0	+ +
" F.....	+ ² +	0 0	+ +	+ ² +	0 0	+ ² +
" L.....	+ +	0 0	+ +	+ +	0 0	+ ² +

The first figure in each column represents phagocytosis, and the second microscopic agglutination.

Summary of the Strains.

These strains may be arbitrarily divided for purposes of discussion into two groups. In the first group are five strains, L, T, R, X, and Ba. Morphologically they were typical pneumococci, with small, though definite capsules. On blood agar the colonies were green, rather small, and dry like those of Group IV pneumococci. All the strains produced a diffuse turbidity in broth and acidified milk. Two of the strains, V and Ba, resembled streptococci in that they were not completely dissolved by bile, did not ferment inulin, and caused

precipitation in glucose ascitic fluid agar. Strain T was not completely bile-soluble, but its reactions otherwise were typical of a pneumococcus. The organisms in this group, except Strain X which was not tested, were relatively avirulent, often failing to kill mice after inoculation with the entire growth of a blood agar slant.

All the organisms were agglutinated macroscopically by Anti-pneumococcus Sera I, II, and III. This agglutination appeared somewhat more slowly than is the case with typical members of the fixed types in their homologous serum, but the clumps of agglutinated bacteria were coarse. The exact titer of agglutination differed with the individual strains. None of the organisms was agglutinated by

TABLE III.

Macroscopic Agglutination in Antipneumococcus Sera of the Various Types.

Strains.	Sera.					
	Type I.	Type II.	Type III.	Subgroup IIa.	Subgroup IIb.	Normal horse.
V	1:64	1:64	1:16	1:8		1:8
T	1:8	1:32	1:16			1:2
R	1:16	1:32		0		1:4
X	1:32	1:64				0
Ba	1:32	1:64	1:4	0	0	0
H	1:64	1:64	1:64	0	0	0
F	1:64	1:64	1:64	0	0	0
L	1:64	1:16	1:16	0	0	0
N	1:32	1:32	1:64	0	0	0

normal horse serum on isolation, but after prolonged cultivation on artificial media slight agglutination appeared in the lower dilutions of horse serum. This tendency could be prevented for a time by frequent passage through animals. The results are given in Table III.

All the strains were phagocyted by normal leucocytes when serum of Type I, II, or III was added to the mixtures. For these organisms an incubation period of 15 minutes was found to give well marked phagocytosis in the test preparations, while the control mixtures with normal horse serum showed no phagocytosis. Longer incubations resulted in the appearance of a slight degree of spontaneous phagocytosis in the controls. Microscopic agglutination was also observed in these preparations with the antipneumococcus sera, and corroboration

rated the results of the macroscopic tests. Table IV shows the results obtained in these tests, which were in most of the cases confirmed by several observations.

It was shown in the case of Strain Ba that a precipitable substance was elaborated in the animal body which caused the formation of a precipitate when the peritoneal washings, cleared by centrifugalization, were added to Antipneumococcus Sera I, II, and III.

Absorption of Antipneumococcus Sera I and II with Strain T removed the agglutinins for Strain T only, and not for the homologous strains, Type I or II, or for the other atypically agglutinable strains

TABLE IV.

Phagocytosis and Microscopic Agglutination in Antipneumococcus Sera of the Various Types.

Strains.	Sera.						
	Type I.	Type II.	Type III.	Subgroup IIa.	Subgroup IIb.	Normal horse.	
V	+ ³ + ³	+ ³ + ³	+ ³ + ³	0 0	0 0	0 0	0 0
T	+ ² + ³	+ ³ + ²	+ ² + ³	0 0	0 0	0 0	0 0
R	+ ² + ³	+ ² + ³	+ ² + ³	0 0	0 0	0 0	0 0
X	+ + ²	+ ² + ³	0 0	0 0	0 0	0 0	0 0
Ba	+ ³ + ³	+ ³ + ³	+ ³ + ³	0 0	0 0	0 0	0 0
H	+ + ²	+ ² + ²	+ ² +	0 0	0 0	0 0	0 0
F	+ ³ + ³	+ ³ + ²	+ ² +	0 0	0 0	0 0	0 0
L	+ +	+ +	+ ² +	0 0	0 0	0 0	0 0
N	+ ³ + ³	+ ³ + ³	+ ² +	0 0	0 0	0 0	0 0

The first figure in each column denotes phagocytosis, and the second microscopic agglutination.

reported here. On the contrary, exhaustion of these sera with the homologous organisms removed, also, all the agglutinins for Strain T and the other strains studied.

In the second group are Strains H, F, L, and N. Morphologically they were typical pneumococci with well developed capsules. The colonies on blood agar were yellowish green, discrete, moderate sized, and fairly moist, resembling more the colonies of pneumococci of Type I or II. All these organisms produced a diffuse turbidity in broth and acidified litmus milk. All the strains were dissolved by bile, and all fermented inulin. None of the strains caused any pre-

precipitation of glucose ascitic fluid agar. In general the virulence of these organisms was greater than that of the preceding group. Strain H killed mice once with a dose of 0.1 cc., and Strain F once with a dose of 0.001 cc. of a 24 hour broth culture. Strains L and N, however, were highly virulent, and killed mice repeatedly in a dose of 0.000001 cc.

All these strains were agglutinated macroscopically by Antipneumococcus Sera I, II, and III, even in a dilution of 1:64. This agglutination took place more slowly than in the preceding group, and the clumps were somewhat smaller. No agglutination occurred in normal horse serum even after long cultivation on blood agar. The results of these tests are given in detail in Table III.

All the strains were phagocyted in the presence of the antipneumococcus sera. Long incubations, from 1 to 2 hours, gave the best results with these strains. No phagocytosis was ever observed in the normal horse serum controls. Microscopic agglutination also occurred in the mixtures, and confirmed the results obtained by the macroscopic method. Table IV shows the results of these tests.

It was shown that all these strains elaborated a soluble substance in the animal body which caused the formation of a precipitate on the addition of serum of Type I, II, or III. No precipitate resulted when normal horse serum was added.

Antipneumococcus Sera I and II were absorbed with the homologous strain, and also with Strain N. The sera exhausted with the homologous strains showed no agglutinative or phagocytic activity towards the homologous strains, or towards any of the strains in this study. On the other hand, the sera (Types I and II) exhausted with Strain N were as active on typical Type I and II organisms, and on all the atypically agglutinable strains with which they were tested, except Strain N, as were the unabsorbed sera. The agglutinins concerned in the reaction are, therefore, to be regarded as minor agglutinins. The behavior of the bacteriotropins was similar to that of the agglutinins.

Strains N and L were of sufficient virulence to permit testing the protective power of the antipneumococcus sera. Both strains killed mice in all doses used down to 0.000001 cc. when normal serum was added to the culture dilutions. With Strain L, Sera I, II, and III

protected all the animals receiving 0.001 cc. or less (1,000 times the minimal lethal dose). With Strain N, Sera I and II protected all the mice receiving 0.01 cc. or less (10,000 times the minimal lethal dose). The homologous immune serum (Strain N) was found to protect mice from 0.5 cc. of the culture (500,000 times the minimal lethal dose).

The results demonstrate that a serum may possess protective power against an organism for which it possesses only minor agglutinins, and are in accord with the findings of Avery (7) in regard to the behavior of atypical Type II organisms with Type II serum.

This protective power for mice suggests the possibility that Type I (or II) serum might have some therapeutic value in cases of human infection with these organisms.

In order to determine whether or not there existed interrelationships between these pneumococci which reacted with Antipneumococcus Sera I, II, and III, an immune serum was prepared to seven of the nine strains studied. The serum having the highest titer was that produced against Strain T. This serum agglutinated the homologous organism in a dilution of 1:2,000.

As far as possible each strain was tested in all the immune sera. The results of phagocytic tests and the presence or absence of microscopic agglutination are shown in Table V, and the results of macroscopic agglutination tests in Table VI. In general, all the strains were serologically distinct, though there seemed to be some interrelationships between certain strains. Strain V was agglutinated and phagocytosed actively in the serum of Strain R, as well as in the homologous serum; and Strain R, similarly, in serum of Strain V. Strains H, F, and N also showed some degree of cross-agglutination and phagocytosis, though with these organisms agglutination and phagocytosis were less active in the heterologous sera than in the homologous serum. The interreactions of Strains H and N were demonstrated also by precipitin tests, and a slight precipitin reaction was also observed with Strain F when tested with Serum H.

These sera caused no agglutination or phagocytosis of typical members of Type I or II, or of Subgroups IIa, IIb, or several other atypical Type II strains which were tested.

A possible relation between the pneumococci agglutinating with Antipneumococcus Sera I, II, and III and the fixed types is suggested by certain changes which occurred with Strain B while it was under observation.

This organism was isolated April, 1916, from a case of diabetes with a terminal lobar pneumonia, by sputum culture, lung puncture, and blood culture. On isolation it was agglutinated macroscopically by Serum II in a dilution of 1:8, while no agglutination occurred with Serum I. No phagocytosis occurred with Serum I or II, though microscopic agglutination was observed in the preparation with Serum II. As shown by Clough (5), this reaction is characteristic of atypical

TABLE V.
Crossed Phagocytic and Agglutinative Reactions.

Strains.	Immune sera.								Normal rabbit serum.							
	V		R		T		Ba			H		F		N		
V	+	+	+	+	0	0	0	0	0	0			0	0	0	0
R	+	+	+	+	0	0	0	0	0	0			0	0	0	0
T	0	0	0	0	+	+	0	0	0	0			0	0	0	0
X	0	0			0	0									0	0
Ba	0	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	+	+	+	0	+	+	0	0
F	0	0	0	0	0	0	0	0	+	+	+	+	+	+	0	0
N	0	0	0	0	0	0	0	0	+	0	+	+	+	+	0	0
L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The first figure in each column denotes phagocytosis, and the second microscopic agglutination.

Type II pneumococci. The organism was considered, therefore, to be an atypical Type II.

This strain was cultivated on blood agar (with three intervening animal passages) for 8 months, and was then tested again. At this time the strain had become agglutinable and phagocyttable in Antipneumococcus Sera I, II, and III. The exact cultural and serological reactions obtained have been described previously (Strain 5). This strain will be referred to as Ba.

Between January and March, 1917, this organism was passed through seven mice. When tested at this time it was still agglutinated macroscopically with Sera I, II, and III and not with normal horse serum. After passage through two more mice it was again tested as to its phagocytability in these sera. No phagocytosis or microscopic agglutination occurred with sera of Type I or III,

or with normal serum. With Serum II no phagocytosis was observed, but there was well marked microscopic agglutination. Tests were then made with a Subgroup IIa serum³ obtained from the Hospital of The Rockefeller Institute, with two specimens of Subgroup IIa serum prepared in this laboratory, and also with the active postcritical sera of two patients with Subgroup IIa infection. Marked phagocytosis and agglutination occurred with all these sera.

A serum was then prepared by immunizing a rabbit against this strain, which will be referred to for convenience as Bb. This Bb serum caused marked phagocytosis and agglutination of the homologous strain, and also of three other Subgroup IIa strains with which it was tested. It caused no phagocytosis or microscopic agglutination of a typical Type I or II strain. Macroscopic agglutinations corroborated the results of microscopic agglutinations. This serum caused no phagocytosis or agglutination of Strain Ba. It seemed clear, then, that Strain

TABLE VI.
Crossed Macroscopic Agglutination Reactions.

Strains.	Immune sera.							Normal rabbit serum.
	V	R	T	Ba	H	F	N	
V	1:256+		0					0
R	1:64	1:256	0		0			0
T	0	0	1:2,048		0			0
X	0		0					0
Ba	0	0	0	1:256+	0	0	0	0
H	0	0	0	0	1:512	0	1:32	0
F	0	0	0	0	1:16	1:64	0	0
N	0	0	0	0	0	0	1:512	0
L	0	0	0	0	0	0	0	0

Bb was a typical Subgroup IIa strain, and differed serologically from Strain Ba. If errors could be excluded, it seemed probable, therefore, that a mutation had occurred.

In order to confirm this finding, and to exclude the possibility of a confusion of strains, Strain Ba was then passed through another series of mice. The strains in this series will be referred to as Ba followed by a figure which indicates the number of mouse passages. After each passage the strain was recovered by plating the heart's blood on blood agar, and subculturing from a single colony to eliminate as far as is possible in this way the chance that we were dealing with a mixture of strains, and so satisfy the pure line requirement.

³ This serum was obtained from the Hospital of The Rockefeller Institute, through the kindness of Dr. Alan M. Chesney.

Serum was obtained by immunizing a rabbit to a strain of this Ba series. This serum caused marked phagocytosis and agglutination of the first Ba strain, and also of the succeeding Ba strains, but was without any effect on the Bb strains.

No change occurred in the strains of this series during the first five animal passages. They were agglutinated macroscopically by the Ba serum, but not by a Subgroup IIa serum. After the sixth mouse passage, however, Strain Ba6 failed to become agglutinated macroscopically by Ba serum, but was agglutinated by a Subgroup IIa serum. This change was corroborated by phagocytic tests, the results of which are tabulated below.

Serological reactions of Strain Ba6.	Phagocytosis.	Microscopic agglutination.
Strain Ba6 + Type I serum.....	0	0
“ Ba6 + “ II “	0	+ ³
“ Ba6 + Ba serum.....	0	0
“ Ba6 + Bb “	+ ³	+ ³
“ Ba6 + Subgroup IIa serum.....	+ ³	+ ³
“ Ba6 + “ IIb “	0	0
“ Ba6 + normal horse and rabbit serum.....	0	0

It seemed probable, then, that the same definite changes in serological reactions previously observed must have again occurred during the sixth mouse passage.

Strain Ba5 was then passed through another mouse to see whether this strain would again change into a Subgroup IIa. The strain then obtained was labelled Ba6a. The reactions of this strain follow.

Serological reactions of Strain Ba6a.	Macroscopic agglutination.	
	2 hrs.	24 hrs.
Strain Ba6a + Ba serum.....	1: 64+	1: 64+
“ Ba6a + Bb “	1: 32	1: 32
“ Ba6a + normal horse serum.....	0	0

Serological reactions of Strain Ba6a.	Phagocytosis.	Microscopic agglutination.
Strain Ba6a + Type I serum.....	+	+ ³
“ Ba6a + “ II “	+ ²	+ ³
“ Ba6a + “ III “	+ ²	+ ²
“ Ba6a + Subgroup IIa serum.....	+ ³	+ ³
“ Ba6a + “ IIb “	0	0
“ Ba6a + Ba serum.....	+ ²	+ ²
“ Ba6a + Bb “	+ ²	+ ²
“ Ba6a + normal horse serum.....	0	0

From these results it seemed likely that Strain Ba6a was a mixture of the two forms, one corresponding to the Ba strains agglutinating with all three antipneumococcus sera, and one corresponding to the Bb (Subgroup IIa) strains. Indeed, in the preparations of Strain Ba6a there was some difference in the morphological appearance of the organisms, those which were agglutinated by the Bb and Subgroup IIa sera being larger, more loosely clumped, and having moderate sized capsules; while those agglutinated by the Ba serum appeared smaller, and the clumps were denser. With the specimen of stain used for these preparations the capsules on the latter organisms were not stained.

This culture Ba6a was plated out, and two types of colonies were fished, one of which grew with a delicate growth on blood agar (called Ba6a-Subgroup IIa form), while the other grew more luxuriantly, and stained the base of a blood agar slant an intense green (called Ba6a-undifferentiated form). These two strains were then tested for phagocytosis and microscopic agglutination in Ba and Bb sera and in Antipneumococcus Sera I and II. The results of the tests follow.

Serological reactions of both forms of Strain Ba6a.	Phagocytosis.	
	Microscopic agglutination.	
Strain Ba6a (undifferentiated form) + Type I serum.....	+	+ ²
“ Ba6a “ “ + “ II “	+	+ ³
“ Ba6a “ “ + Ba serum.....	+ ³	+ ³
“ Ba6a “ “ + Bb “	0	0
“ Ba6a “ “ + normal horse serum.....	0	0
“ Ba6a (IIa form) + Type I serum.....	0	0
“ Ba6a (IIa “) + “ II “	0	+ ³
“ Ba6a (IIa “) + Ba serum.....	0	0
“ Ba6a (IIa “) + Bb “	+ ³	+ ³
“ Ba6a (IIa “) + normal horse serum.....	0	0

The results of the tests with Sera I and II were confirmed by macroscopic agglutinations.

Serological reactions of both forms of Strain Ba6a.	Macroscopic agglutination.	
	1 hr.	24 hrs.
Strain Ba6a (undifferentiated form) + Type I serum.....	1:8	1:16
“ Ba6a “ “ + “ II “	1:64	1:64
“ Ba6a “ “ + normal horse serum.....	0	0
“ Ba6a (IIa form) + Type I serum.....	0	0
“ Ba6a (IIa “) + “ II “	0	1:16
“ Ba6a (IIa “) + normal horse serum.....	0	0

Furthermore the reaction of these strains with inulin serum water differed, Strain Ba6a (IIa form) fermenting it with the production of acid and coagulation, while Strain Ba6a (undifferentiated form) did not ferment it.

To summarize the changes, on artificial cultivation an atypical Type II pneumococcus (B original) changed in its serological reactions into one of the atypically agglutinable organisms (called Ba) reacting with Antipneumococcus Sera I, II, and III. That this change was not due to a confusion of strains was proved by testing Ba serum with all the other strains in the laboratory reacting in this way, and also by testing the Ba organism with the immune sera produced against all the atypically agglutinable strains. No inter-reactions occurred.

After animal passage Strain Ba changed back abruptly into a strain, Bb, having the phagocytic and agglutinative reactions of a Subgroup IIa. These two strains, Ba and Bb, were serologically distinct, and immune sera produced against each had no effect on the other strain.

In order to confirm this finding, Strain Ba was again passed through a series of mice, and again changed abruptly into a Subgroup IIa form. From the last culture in this series reacting with the Ba and not with the Bb serum, a third series of mice was started in an attempt to produce again a mutation. A change occurred during the next animal passage, and the strain recovered from this mouse was shown to be a mixture of the a and b forms. These forms were separated by plating. The a form reacted with the Ba serum and also with Antipneumococcus Sera I and II, but not with the Bb serum; while the b form reacted with the Bb serum, and with Subgroup IIa serum, but not with the Ba serum. It was not acted on by Type I serum, and was agglutinated but not phagocyted in Type II serum. The morphological and cultural reactions of these strains also differed. The a form had small capsules, grew profusely, and colored diffusely the base of a blood agar slant an intense green, did not ferment inulin, and was only partially soluble in bile. The b form had well developed capsules, grew delicately with no diffuse staining of the blood agar, fermented inulin, and was completely soluble in bile. It seemed definite, then, that a mutation of this organism had occurred.

Mutations of various species of bacteria have been noted by a number of observers. Changes in the morphological and cultural characteristics of streptococci and pneumococci which were regarded as mutations of one species into the other have been observed by Buerger and Ryttenberg (8), Schereschewsky (9), Davis (10), Aschner (11), and others.

Rosenow (12) has reported not only cultural and morphological changes indicating mutations between *Streptococcus mucosus*, pneumococcus, *Streptococcus viridans*, *Streptococcus rheumaticus*, and *Streptococcus hemolyticus*, but also mutations of one type of pneumococcus into another based on serological reactions. A typical Type I pneumococcus was converted by animal passage into a Type II pneumococcus, and again into a hemolytic streptococcus. Likewise streptococci were transformed into Type I and II pneumococci. A Pneumococcus Type I sent from The Rockefeller Institute was agglutinated by Serum I when grown in all media except ascites dextrose agar, but when grown in this medium was agglutinated only by Serum II. A Pneumococcus Type II from the same source was agglutinated specifically when grown on ascites dextrose agar, but was agglutinated by both Sera I and II when grown on other media.

Such mutations from one type of pneumococcus to another have not been confirmed by others. Cole (13) states that in the study of several hundred strains of pneumococci, during which some have been under artificial cultivation for 3 years, and some have been passed through as many as 100 animals, no transformations have been noted.

Our experience also indicates that, while slight variations in cultural characteristics such as bile solubility and inulin fermentation are not unusual, such mutations as Rosenow described are not of frequent occurrence. Several Type I and atypical Type II strains, which have been under artificial cultivation for 4 years, one Type II strain for 3 years, and several Type III strains for 2 years have shown no changes in their cultural or serological reactions. It is possible, however, that pneumococci which are less strictly parasitic, and possibly less highly differentiated, may be more susceptible to mutation than are the fixed types.

Although no further proof has been obtained on this point, this mutation of Strain B suggests that these pneumococci reacting with antipneumococcus sera of all three types may represent primitive, relatively undifferentiated forms of pneumococci from which the fixed types may have arisen.

SUMMARY.

In this paper are reported the results of a study of nine strains of pneumococci agglutinating with antipneumococcus sera of all three types (Nos. I, II, and III). Seven of the strains were the cause of serious or fatal infections in human beings.

Morphologically they were typical pneumococci with characteristic growth on ordinary media. Most of the strains were soluble in bile, fermented inulin, and caused no precipitation on glucose ascitic fluid agar. Two of the strains, however, resembled streptococci in these three cultural characteristics, but have been regarded as pneumococci on account of their serological reactions. Variations in the cultural reactions occurred with several strains while they were under observation.

The virulence of the strains varied greatly, some strains being almost non-pathogenic, and others killing mice in doses of 0.000001 cc. of a 24 hour broth culture.

Antipneumococcus Sera I, II, and III agglutinated all the strains in fairly high dilution (1:8 to 1:64 or higher), while normal horse serum caused no agglutination.

Antipneumococcus Sera I, II, and III stimulated active phagocytosis of all the strains, while no phagocytosis occurred in control preparations with normal horse serum.

These strains elaborated a soluble substance in the body of inoculated mice which caused the formation of a precipitate when the peritoneal washings, cleared by centrifugalization, were added to the antipneumococcus sera of all three types.

Antipneumococcus Sera I, II, and III protected mice equally well against 1,000 to 10,000 times the minimal lethal dose of the two strains with which protection tests could be carried out.

Absorption of serum of Types I and II with the homologous pneumococcus removed the agglutinins and the bacteriotropins for all

these strains. Absorption of these sera with Strains T and N removed the agglutinins and the bacteriotropins for the homologous strain only, and not for typical members of Type I or II, or for the other atypically agglutinable strains reported in this paper. The agglutinins concerned in the agglutination of these peculiar strains are therefore minor agglutinins.

As shown not only by agglutination tests, but also by protection tests and agglutinin absorption tests, these organisms bear the same relation to Types I, II, and III, as do atypical Type II strains to Type II.

Immune sera were prepared with these strains, and each strain was tested with all the immune sera by means of phagocytic and agglutinative reactions. In general, the strains were found to be serologically distinct, though some interrelationships existed between Strains V and R, and between Strains H, F, and N. These sera had no activity towards strains belonging to Type I or II, or atypical Type II.

A mutation occurred in one of the strains, B, while it was under observation. On isolation this strain had the cultural reactions of a typical pneumococcus, and had the phagocytic and agglutinative reactions of an atypical Type II. After 6 months cultivation on blood agar its serological reactions changed, and it became actively phagocyted and agglutinated in antipneumococcus sera of Types I, II, and III. Its cultural characteristics also changed, and it became bile-insoluble, did not ferment inulin, and caused precipitation in glucose ascitic fluid agar. At this time it caused an intense green discoloration at the base of the blood agar slants around the water of condensation. By repeated animal passages this strain was three times made to revert abruptly to its original form (atypical Type IIa), both in cultural and serological reactions. An immune serum was prepared to each form of the strain, and each serum acted strongly on the homologous form, but was without action on the heterologous form of the strain.

This mutation suggests that these pneumococci reacting with all three types of antipneumococcus sera may represent primitive, relatively undifferentiated forms from which the fixed types may have arisen.

BIBLIOGRAPHY.

1. Mathers, G., The varieties of pneumococci causing lobar pneumonia, with especial reference to their biologic differences, *J. Infect. Dis.*, 1915, xvii, 514.
2. Wollstein, M., and Benson, A. W., Types of pneumococcus found in the pneumonias of infants and young children, *Am. J. Dis. Child.*, 1916, xii, 254.
3. Blake, F. G., Methods for the determination of pneumococcus types, *J. Exp. Med.*, 1917, xxvi, 67.
4. Stryker, L. M., Variations in the pneumococcus induced by growth in immune serum, *J. Exp. Med.*, 1916, xxiv, 49.
5. Clough, P. W., Phagocytosis and agglutination in the serum in acute lobar pneumonia; the specificity of these reactions, and the regularity of their occurrence, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 167.
6. Dochez, A. R., and Avery, O. T., The elaboration of specific soluble substance by pneumococcus during growth, *J. Exp. Med.*, 1917, xxvi, 477.
7. Avery, O. T., A further study on the biologic classification of pneumococci, *J. Exp. Med.*, 1915, xxii, 804.
8. Buerger, L., and Ryttenberg, C., Observations upon certain properties acquired by the pneumococcus in the human body, *J. Infect. Dis.*, 1907, iv, 609.
9. Schereschewsky, L., Streptokokken und Pneumokokken und ihr gegenseitiges Verhältnis, *Centr. Bakteriöl., 1te Abt., Orig.*, 1909, xlix, 72.
10. Davis, D. J., Interrelations in the streptococcus group with special reference to anaphylactic reactions, *J. Infect. Dis.*, 1913, xii, 386.
11. Aschner, P. W., Studies on pneumococci and streptococci, *J. Infect. Dis.*, 1917, xxi, 409.
12. Rosenow, E. C., Transmutations within the streptococcus-pneumococcus group, *J. Infect. Dis.*, 1914, xiv, 1.
13. Cole, R. I., Pneumococcus infection and immunity, *N. Y. Med. J.*, 1915, ci, 1.

STUDIES OF OXYGEN IN THE VENOUS BLOOD.

V. DETERMINATIONS ON PATIENTS WITH ANEMIA.

By CHRISTEN LUNDSGAARD, M.D.

(From the Medical Clinic of the University of Copenhagen, Copenhagen, Denmark.)

(Received for publication, May 23, 1919.)

In the first four articles of this series (1-4) facts were discussed which indicate that the oxygen content of the venous blood depends on a number of factors: (1) the total oxygen-combining power, or the hemoglobin content of the blood; (2) the degree of oxygen saturation of the blood in the lungs; (3) variations in the metabolism of the tissues drained by the vein tapped, as compared with the metabolism of the rest of the body; (4) variations in the rate of blood flow through the tissues drained compared with the rest of the body; (5) variations in the minute volume of the heart.

In order to find the effect of one of these factors on the oxygen content of the venous blood, it is necessary to control the other factors or keep them constant.

In Papers II, III, and IV we studied in cardiac patients the effect of the fifth of the above factors. It was not possible to exclude completely the effects of the other four factors, but by choosing suitable subjects, *viz.* decompensated cardiac patients and normal controls, and by excluding the other factors as nearly as possible, or by correcting for them, we were able to observe with a certain degree of accuracy the effect of the cardiac output on the venous oxygen. In particular, variations in the hemoglobin content were corrected for by basing our conclusions not on the absolute value of the venous oxygen, but on the oxygen unsaturation, or the difference between the venous oxygen and the total oxygen capacity of the blood. The correctness of following this procedure in individuals with varying hemoglobin content was assumed rather than exactly proved.

This paper presents the proof in a study of the venous oxygen content and unsaturation in a series of patients in whom the hemoglobin varied over a wide range.

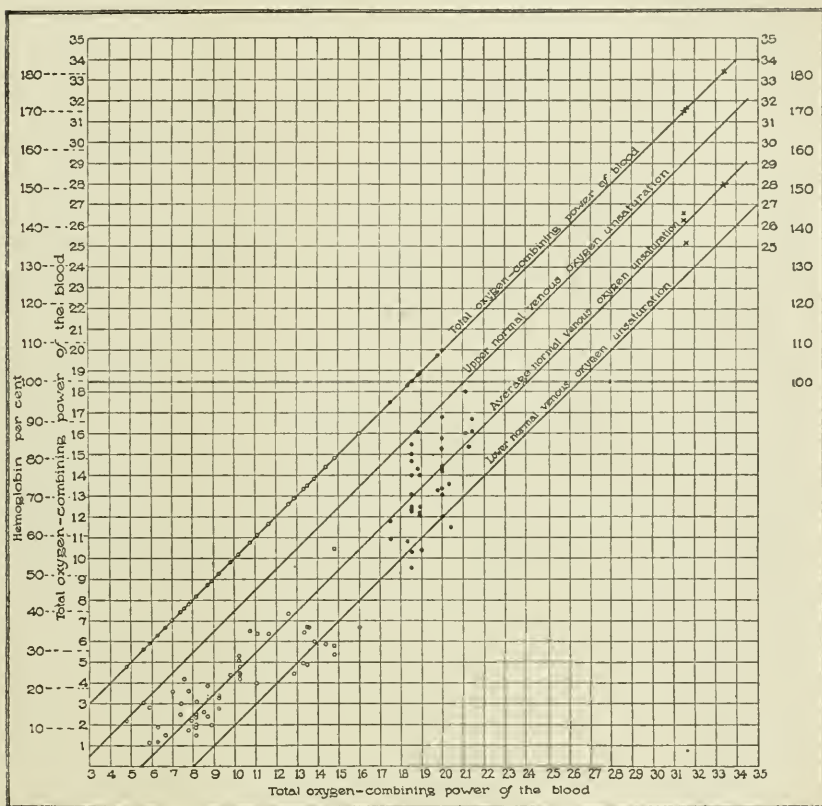
Nine patients with anemia and one patient with polycythemia¹ were selected for this purpose. The hemoglobin in these cases varied from 181 to 27 per cent. The oxygen-combining power of the blood varied consequently from 33.40 to 5.00 volumes per cent. The lungs were examined before each determination and did not show any pathological changes, which might influence the saturation of the blood. The blood samples were drawn at least 2 hours after a meal and after 10 minutes rest in bed. Nothing was found to indicate any abnormality in the circulation, apart from a slight increase in the pulse rate in some of the patients. The diuresis and the blood pressure were found to be normal.

The technique in drawing the blood samples was exactly the same as described in Paper I of this series. In determining the oxygen of the venous blood Van Slyke's method was used (5). The total oxygen-combining power of the blood was either determined directly—Van Slyke's method—or calculated from the hemoglobin determined by Haldane's method.

The results on the patients with anemia are given in Tables I to X. As a whole, the values of the oxygen in the venous blood increase proportionally with the increase in the hemoglobin, giving values for the oxygen unsaturation, which vary within the ordinary normal limits—2.5 and 8 volumes per cent. In a few instances the unsaturation has been between 8 and 10 volumes per cent. This was found only in patients in which hemoglobin had increased rather quickly from low values to about the normal. In other words, the amount of oxygen taken away from the blood during passage through the body capillaries in resting individuals is practically independent of the hemoglobin content of the blood. In order to make the relations more clear the results are presented in Text-fig. 1, where the abscissæ and the ordinates indicate oxygen-combining power in volumes per cent and hemoglobin in per cent. The corresponding percentages are also indicated, as calculated on Haldane's average, 18.5 per cent oxygen equalling 100 per cent hemoglobin. On the upper oblique line are the values for the total oxygen-combining power in each given instance

¹ The details on this patient will be published in the next number of this *Journal*, but the values for unsaturation are given in Text-fig. 1 of this paper.

and on the ordinate belonging to this point is indicated the value for the oxygen of the venous blood; the distance from this figure to the base-line gives the venous oxygen content, while the distance from the point to the upper oblique line measures the venous unsaturation.



TEXT-FIG. 1. Values for the total oxygen-combining power and the venous oxygen in individuals with different amounts of hemoglobin. The circles with light centers indicate values for anemic patients, the solid black circles values for normal individuals, and the crosses values for a polycythemic patient.

The values of the oxygen-combining power lie, of course, on a straight line which goes through the zero point at an angle of 45° with the base-line. If the oxygen unsaturation were absolutely constant the values for the oxygen of the venous blood would also lie on a

straight line, which would be parallel to and below the upper one. This is, however, not to be expected. Even in normal individuals, as previously shown, rather extensive variations occur. On the diagram the results reported in the present paper are combined with those obtained in a series of normal individuals and published in Paper I. The earlier figures cover the middle area between the anemic patients and the polycythemic patient reported below. The diagram shows that it was justifiable to use the oxygen unsaturation in order to exclude the influence of variations in the hemoglobin content of the blood. The variations in the oxygen unsaturation in these cases are probably due to small variations in one or several of the other previously named factors. Of these, oxygenation in the lungs is probably the most constant (in these patients), the minute volume and the local blood flow probably the most variable.

These results are of interest not only for the interpretation of oxygen determinations in general, but also for the understanding of the circulatory mechanism in patients with anemia.

The fact that the hemoglobin is the only bearer of oxygen from the lungs to the tissues has made it a question of moment in what way anemic patients are able to compensate for the decrease in oxygen-combining power of the blood. For a considerable period of time it was supposed that no compensation was necessary, for the reason that the total metabolism was considered to be lower in anemic patients. This theory was based on experiments on dogs by Bauer (6) and on conclusions from fatty degenerations in the organs of patients dying from anemia, the fat being considered the result of lack of oxygen. Experiments by Kraus (7) and others showed that the metabolism is not diminished in patients with anemia. This being the case, a compensatory mechanism for the lack of hemoglobin was thought necessary.

Three theories were put forward: (*a*) an increased oxygen capacity of the hemoglobin—increased “specific capacity” (Hoppe-Seyler (8), Bohr (9), and his pupils), (*b*) an increase in the blood flow, (*c*) an increased percentile consumption of the oxygen in the blood. After Butterfield (10), Barcroft and Morawitz (11), Masing and Siebeck (12), and von Reinhold (13), who proved the proportionality between the color index and the oxygen-combining power of the hemoglobin, the theory about the “specific oxygen capacity” is untenable.

From the experimental data in the literature it is impossible to decide between the other two theories. In some experiments the blood flow has been found increased (Kraus (14) and Plesch (15)). Other experiments are interpreted as showing increased consumption; for instance, experiments of Finkler (16). Morawitz and Roehmer, who have determined the oxygen in the venous blood in eighteen patients with anemia and one with polycythemia, interpret their results to indicate that both methods of compensation are used by the anemic organism.

In discussing the problem it is evidently necessary to distinguish between the resting and working organism. In a resting organism the rate of oxygen consumption is approximately a constant for each particular individual, and for different individuals within the same species it is constant except for variations caused by differences in the surface and weight of the body. The increase caused by exercise is, of course, dependent on the extent of the work. From the determination of the minute volume of the heart and the oxygen unsaturation of the venous blood, we know that the normal resting organism on the average uses 5.5 volumes per cent oxygen in the capillaries. The normal oxygen capacity of the blood being 18.5 volumes per cent, the normal individual consequently has 13 volumes per cent of unused oxygen in his veins. This amount of oxygen may be termed the reserve oxygen, and looked upon as analogous to the reserve force of the heart. When the organism for one reason or another needs more oxygen, or when the blood flow in the capillaries is slower than normally, the organism uses the reserve oxygen. Anemia is in this respect simply a condition where the amount of reserve of the blood is diminished in the same degree as the hemoglobin; the opposite takes place in polycythemia. From a purely mechanical point of view it is clear that in a resting individual no compensation is needed until the hemoglobin has fallen so low that all the reserve oxygen is used in each circulation. This occurs when the oxygen-combining power of the blood has fallen to 5.5 volumes per cent, which corresponds to 30 per cent hemoglobin. Above this value no compensation seems necessary; below this the organism must compensate for the lack of reserve oxygen storage in the blood, and this can be done only in one way, by an increased output of the heart.

The results of the determination in this paper show that this viewpoint is as a whole correct. The last residuum of the oxygen in the blood—the last part of the reserve oxygen—is taken away by the body cells in resting individuals just as easily as the first part. This, of course, is of great importance for the body, especially for the heart.

Clinical experience agrees with the experimental results in this paper; a bodily and mentally resting anemic patient usually does not show any marked reaction from the circulation, until the hemoglobin percentage has fallen below about 30. In patients whose hemoglobin is below that value we usually find an increased pulse rate and palpitation of the heart even at rest.

SUMMARY.

1. Determinations of the oxygen content and the oxygen unsaturation of the venous blood have been performed on patients with varying amounts of hemoglobin.

2. The oxygen unsaturation of the venous blood is independent of the oxygen capacity, unless the latter is reduced below the normal value for oxygen unsaturation (about 5 volumes per cent). In a polycythemic patient,¹ for example, with 33.4 volumes per cent oxygen capacity (181 per cent hemoglobin), the venous oxygen content was 28 volumes per cent, giving an unsaturation of 5.4 volumes per cent. Similarly, in an anemic patient with only 6.7 volumes per cent oxygen capacity (36 per cent hemoglobin), the venous oxygen was 1.5, giving an unsaturation of 5.2 volumes per cent. This means that the tissues extract from the blood all the oxygen they need with apparently equal readiness, regardless of whether the extraction leaves a great oxygen reserve in the blood as in polycythemia, or practically no reserve as in anemia.

3. The results seem to show that the resting organism does not increase its circulation until all the reserve oxygen is used. This means that the resting anemic organism does not need or use any compensation for its anemia until the hemoglobin has sunk below 30 per cent. Below that value the organism increases the blood flow in order to secure to the tissues the normal amount of oxygen.

TABLE I.

Oxygen Unsaturation of the Venous Blood in a Patient with Severe Anemia.

Housewife, age 38 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	July 12	4.40	4.00	4.20	41	7.58	3.38	98	22
2	" 18	3.54	3.64	3.59	38	7.03	3.44	96	20
3	Aug. 3	3.04		3.04	40	7.41	4.37	96	22
4	" 14	1.62	1.34	1.48	44	8.15	6.67	92	22
5	" 22	2.17	2.53	2.35	44	8.15	5.80	90	20
6	" 25	3.08	3.08	3.08	44	8.15	5.07	90	22
7	Sept. 9	2.53		2.53	44	8.15	5.62	90	22
8	" 14	1.75	2.21	1.98	44	8.15	6.17	92	20
9	" 21	1.74	2.20	1.97	48	8.88	6.91	86	20
10	Oct. 5	3.96	3.96	3.96	50	9.25	5.29	90	18
11	" 19	4.01	4.93	4.47	53	9.82	5.35	90	16

TABLE II.

Oxygen Unsaturation of the Venous Blood in a Patient with Severe Anemia.

Housewife, age 41 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	Aug. 29	4.63	5.54	5.09	55	10.20	5.11	66	18
2	Sept. 3	6.46	6.46	6.46	75	13.87	7.40	60	16

TABLE III.

Oxygen Unsaturation of the Venous Blood in a Patient with Severe Anemia from Hematemesis.

Male, engineer, age 20 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	Oct. 29	1.19		1.19	34	6.30	5.11	72	24
2	Nov. 5	1.27	1.05	1.16	32	5.93	4.77	80	18
3	" 14	2.20	2.20	2.20	43	7.96	5.76	62	18
4	Dec. 3	1.70	2.15	1.93	44	8.15	6.22	84	18
5	" 15	3.32		3.32	50	9.25	5.93	90	22

TABLE IV.

Oxygen Unsaturation of the Venous Blood in a Patient with Severe Anemia from Metrorrhagia.

Housewife, age 47 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	Aug. 3	2.82		2.82	50	9.25	6.43	86	26
2	" 13	4.86	5.66	5.26	55	10.17	4.91	86	24
3	" 19	3.95	3.95	3.95	60	11.09	7.14	88	24
4	" 27	5.84	6.76	6.40	63	11.65	5.25	86	20
5	Sept. 9	6.68	6.68	6.68	73	13.50	6.82	86	20

TABLE V.

Oxygen Unsaturation of the Venous Blood in a Patient with Severe Anemia from Hemorrhagic Colitis.

Male, workman, age 33 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	Sept. 19	1.73	1.73	1.73	42	7.78	6.05	76	24
2	" 25	4.54		4.54	55	10.27	5.78	80	24
3	Oct. 8	4.75	4.99	4.87	55	10.27	5.40	68	20
4	" 31	5.92	5.92	5.92	75	13.87	7.95	76	20

TABLE VI.

Oxygen Unsaturation of the Venous Blood in a Patient with Severe Anemia from Miscarriage.

Housewife, age 26 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	Nov. 19	2.21	2.21	2.21	26	4.81	2.60	100	24
2	Dec. 4	3.05		3.05	30	5.55	2.50	112	24
3	" 8	3.87	3.87	3.87	43	8.70	4.93	100	26

TABLE VII.

Oxygen Unsaturation of the Venous Blood in a Patient with Severe Anemia from Hematemesis.

Male, carpenter, age 39 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	Sept. 17	3.56	3.56	3.56	42	7.78	4.22	60	15
2	" 24	3.11	3.57	3.34	50	9.25	5.91	60	14
3	Oct. 3	4.07	4.97	4.52	55	10.27	5.75	60	14
4	" 12	4.04	4.86	4.45	55	10.27	5.82	60	14
5	Nov. 11	4.22		4.22	55	10.27	6.05	72	14
6	" 14	6.30	6.70	6.50	58	10.73	4.23	60	15

TABLE VIII.

Oxygen Unsaturation of the Venous Blood in a Patient with Severe Anemia.

Housewife, age 44 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	Nov. 14	2.20	3.10	2.65	46	8.52	5.97	80	20
2	Dec. 29	10.55	10.35	10.45	80	14.80	4.35	76	19

TABLE IX.

Oxygen Unsaturation of the Venous Blood in a Patient with Pernicious Anemia.

Male, porter, age 44 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	Aug. 13	7.46	7.38	7.42	68	12.57	5.15	72	22
2	" 21	4.90	4.90	4.90	73	13.50	8.60	84	24
3	" 28	5.86	5.96	5.92	78	14.42	8.50	82	22
4	Sept. 3	5.82	5.82	5.82	80	14.78	8.86	82	20
5	" 10	5.42	5.42	5.42	80	14.78	9.36	72	18
6	Oct. 14	6.70		6.70	87	16.03	9.33	70	18
7	Dec. 14	9.85	10.41	10.13	93	17.20	7.07	80	16

TABLE X.

Oxygen Unsaturation of the Venous Blood in a Patient with Pernicious Anemia.

Male, cab driver, age 53 years.

Determination No.	Date.	Oxygen content of venous blood.			Hemoglobin (Haldane's method).	Calculated oxygen capacity (a).	Oxygen unsaturation (a-v).	Pulse.	Respirations per min.
		Sample 1.	Sample 2.	Average (v).					
	1918	vol. per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent		
1	Aug. 3	2.82	2.82	2.82	32	5.92	3.10	70	30
2	" 13	2.61	2.43	2.52	40	7.41	4.89	66	20
3	" 25	1.63	1.37	1.50	36	6.67	5.17	68	20
4	Sept. 3	2.06		2.06	27	5.00	2.94	84	24
5	" 10	2.17	1.69	1.93	34	6.30	4.37	70	20
6	" 23	2.65	2.19	2.42	47	8.70	6.28	66	20
7	Oct. 5	6.31	6.59	6.40	60	11.09	4.69	60	20
8	" 19	4.46		4.46	70	12.94	8.48	60	20
9	Nov. 18	4.98	4.90	4.94	72	13.31	8.37	64	18
10	Dec. 16	7.17	6.27	6.72	74	13.58	6.86	60	20

BIBLIOGRAPHY.

1. Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxiii, 133.
2. Lundsgaard, C., *J. Exp. Med.*, 1918, xxvii, 179.
3. Lundsgaard, C., *J. Exp. Med.*, 1918, xxvii, 199.
4. Lundsgaard, C., *J. Exp. Med.*, 1918, xxvii, 219.
5. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
6. Bauer, J., *Z. Biol.*, 1872, viii, 567.
7. Kraus, F., *Z. klin. Med.*, 1893, xxii, 449.
8. Hoppe-Seyler, F., *Z. physiol. Chem.*, 1889, xiii, 477.
9. Bohr, C., *Skand. Arch. Physiol.*, 1892, iii, 101.
10. Butterfield, E. E., *Z. physiol. Chem.*, 1909, lxii, 173.
11. Barcroft, J., and Morawitz, P., *Deutsch. Arch. klin. Med.*, 1908, xciii, 223.
12. Masing, E., and Siebeck, R., *Deutsch. Arch. klin. Med.*, 1910, xcix, 130.
13. von Reinbold, B., *Centr. ges. Physiol.*, 1910, v, 689, 721.
14. Kraus, F., *Bibliot. Med.*, 1897, iii, 12.
15. Plesch, J., *Z. exp. Path. u. Therap.*, 1909, vi, 380.
16. Finkler, D., *Arch. ges. Physiol.*, 1875, x, 368.

THE STREPTOCOCCI OF EQUINES.*

By F. S. JONES, V.M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

PLATE 12.

(Received for publication, May 12, 1919.)

INTRODUCTION.

Among the domestic animals no species is apparently more susceptible to infection with streptococci than the horse. In at least two diseases of the respiratory tract these microorganisms play a considerable part either as primary or secondary invaders. Streptococci are also responsible for many wound infections in horses and particularly in foals. Aside from a specific relationship to equine diseases, the relationship of the pathogenic streptococci found in horses to those of man is worthy of careful study.

Schütz (1) and Chantemesse and Delamotte (2) observed in the pneumonic areas of the lungs of horses suffering from influenza diplococci which when cultivated in bouillon developed as chains. Throughout the literature these streptococci are frequently referred to as the diplococcus or bicoccus of pneumonia.

Among the earlier workers Schütz (3), Baruchello (4), Sand and Jensen (5), and Poels (6) isolated or observed long chained streptococci in the nasal discharges and pharyngeal abscesses of horses suffering from strangles (adenitis equorum).

The earlier workers described the morphologic and cultural characters as they occurred on a few simple media such as agar, coagulated serum, and in bouillon and they attempted to show specific differences between these two streptococci. Their pathogenicity for mice and rabbits was clearly pointed out.

Galtier and Violet (7) also succeeded in isolating cocci in chains from the lungs of horses which had died of influenza. They observed that the length of chains varied widely in different tissues and cultures. In attempting to ascertain the source of the organism, they examined the nasal and pharyngeal mucosa

* This study was conducted during the war as a part of a study of infections occurring among horses, as a measure of military importance.

and feces of normal horses. They came to the conclusion that the specific streptococcus existed as a saprophyte in all these places. The characters on which the identifications were made were insufficient to separate streptococci into the various groups.

Lignières (8) isolated streptococci from the lungs, pleural fluid, liver, spleen, kidneys, and blood of horses dying of influenza. The cocci were usually ovoid and retained the stain by Gram's method. They grew as flocculi in bouillon and produced acid. Milk was usually coagulated in 24 to 48 hours. His cultures were pathogenic for mice and rabbits but guinea pigs were more resistant. 2 cc. of a bouillon culture injected subcutaneously into a horse produced an abscess. He compared Schütz's pneumonia diplococcus with Galtier and Violet's and his own streptococci and believed they were of the same species. He then undertook to show that Schütz's strangles streptococcus was identical with those isolated from influenza. Inasmuch as morphology and pathogenicity seemed to be the principal characters on which the comparisons were made, the results were not clear, although he regarded the streptococci as the same species. For a time Lignières was inclined to believe that streptococci were the cause of influenza but later he succeeded in isolating a rod-shaped organism of the septicemia hemorrhagica group which he claimed to be the agent. A horse inoculated with this culture died; not only was the bacterium recovered from the tissues but a streptococcus as well.

Ostertag (9) states that the nasal secretion of normal horses contains a mixture of bacteria including pus streptococci. He believed that these pus streptococci were identical with those found in influenza, but were not the cause of the disease since intranasal inoculation of material (nasal discharge) containing them failed to produce influenza.

During the next few years many writers discussed these organisms and their relationship to the diseases with which they were associated; in attempts to differentiate them, their virulence for mice and rabbits, the character of the growth in bouillon, and the length of chains proved as inconclusive as formerly. Thus Pfeiler (10) showed that the streptococci isolated from pyogenic conditions and pneumonia grew abundantly throughout bouillon. Bouillon cultures produce septicemia in mice. The strangles streptococcus grew more sparsely in bouillon and left the medium clear. Mice developed pyemia after subcutaneous injections. 50 to 60 cc. of a 24 hour bouillon culture of the pneumonia streptococcus injected intravenously into horses produced a severe reaction which usually terminated in pneumonia or pleuritis. In addition he found agglutinins for the streptococcus in the blood of horses suffering from influenza. He concluded that it was the cause of influenza.

Todd (11) described cocci occurring in chains of 30 to 60 individuals which he isolated from strangles abscesses. Microscopic examination of the nasal discharges usually revealed pairs and short chains. In bouillon they grew at the bottom of the tube, leaving the medium clear. Milk was coagulated in 6 or 8 days. Mice were exceedingly susceptible, rabbits more resistant. Horses when

inoculated subcutaneously with bouillon cultures developed abscesses. Cultures injected into the jugular vein caused only a slight general reaction. From his studies he does not believe that the causative streptococci are carried on the mucosa of the upper air passages of normal horses, but points out that they may be harbored for long periods after recovery.

Jensen (12) in discussing the specific prophylaxis and therapy of the streptococcic diseases of animals touches on the relationship of various streptococci in strangles, influenza, petechial fever, and wound infections. He considers that Holth (13) described a streptococcus (*Streptococcus equi*) which is the cause of strangles. It produces acid in dextrose, maltose, saccharose, and salicin but fails to ferment lactose, raffinose, inulin, or mannite, thus differing sharply from the diplococcus of influenza (*Brustseuchekokkus*) and the pyogenic streptococci. Jensen considers the etiology of influenza still in doubt but believes that the diplococci associated with it belongs to the *Streptococcus pyogenes* group. They differ from *Streptococcus equi* in their ability to ferment lactose and sorbite (Holth).

Bemelmans (14) brings forth a clinical observation to support the claim that strangles and influenza are not due to the same streptococcus. Thus cavalry horses that had recovered from strangles 9, 11, and 45 days previously came down with influenza. He argues that if the infections were caused by the same streptococcus the horses would not have been susceptible to a second attack within such a short period. Bemelmans also quotes Holth, who showed that the strangles organism was an encapsulated streptococcus which fermented dextrose, saccharose, maltose, and salicin but failed to break down lactose, raffinose, inulin, or glycerol.

Koch and Pokschischewsky (15) compared the human streptococcus, *erysipelatos*, with various strains of horse streptococci. The human types had been isolated by the writers. The horse streptococci, with the exception of three strains, had been isolated from cases of strangles in other laboratories. They had been grown on artificial media for long periods (1 to 5 years). The details concerning the more important biological characters are given in twenty instances. All the human strains fermented dextrose, saccharose, maltose, and mannite. Three failed to ferment lactose; the others acted upon this substance. All the horse strains produced acid in dextrose, saccharose, and maltose. Raffinose and mannite were not acted upon. Five strains fermented lactose; the others failed to do so. Both the human and equine streptococci produced hemolysis in blood agar plates. The equine types had a tendency to produce larger areas of hemolysis. The net acid production after several days incubation when measured with 0.05 N sodium hydroxide with phenolphthalein as indicator failed to show marked differences between the horse and human strains. Four strains of each type when grown in dextrose and titrated after maximum acid production had been reached averaged for the human +2.55 per cent and the equine +2.65. After studying the relative virulence of each type they concluded that the streptococcus of strangles is closely related to human *Streptococcus erysipelatos*. It is

interesting to observe that they assumed that the horse streptococci were all of one species whereas in reality there were two, one capable of fermenting lactose and the other unable to do so. The same holds true with the human types. The mannite fermentation by all the human strains was not regarded as of particular significance.

To Schofield (16) we are indebted for a clear description of the streptococci occurring in septic arthritis of foals in Canada. In fifteen cases he found streptococci in the affected joints. The cocci are described as growing in pairs on media other than bouillon. They stain by Gram's method. To determine hemolysis defibrinated rabbit blood, 1 cc., and agar, 10 cc., were poured into Petri dishes and allowed to congeal; the surface was then streaked with culture. Type II produced a clear space 5 to 6 mm. wide about the growth. The clear area extended to the bottom of the dish. In Type III he found that hemolysis took place immediately surrounding the growth but left a discolored zone between the hemolyzed area and the surrounding medium. Apparently the only differences between Types II and III were the size of the hemolytic area and the pathogenicity for rabbits. Type I is spoken of as the Schütz streptococcus but, since his protocols fail to show its presence in the joints or give any data concerning it and since no description of it is offered, it might as well have been omitted. Both Types II and III ferment dextrose, lactose, saccharose, and salicin but fail to ferment inulin, raffinose, dulcitol, and mannitol. He states: "The quantity of acid produced varied with the same strains under different conditions and with similar strains under the same conditions." It is pointed out, however, that no strain lost or acquired fermentative power.

Mathers (17) cultivated streptococci from the nasal discharges of horses suffering from influenza. In seven of twenty-two blood cultures streptococci developed. In addition he found them in exudate from the eye, the pleura, in purulent joints, and involved lymph nodes. Hemolytic streptococci were the only organisms occurring with any degree of regularity. On standard blood agar plates (1 cc. of defibrinated goat blood and 9 cc. of agar) the colonies were round, moist, and adherent. They were surrounded by a clear area of hemolysis 2 to 4 mm. in diameter. The morphology varied; pairs, chains of pairs, and chains were recorded. Many were Gram-negative. They fermented dextrose, lactose, saccharose, and salicin. Milk was acidulated but not coagulated. It is stated that many variations in the fermentative characters occurred. The streptococci were not highly pathogenic for rabbits but highly virulent for horses. Intranasal inoculation of a normal horse with "extracts" prepared from the nasal discharge of a horse suffering from influenza resulted in a "typical attack" of the disease. To test Gaffky and Lührs' (18) findings that influenza is caused by a filterable agent, a series of sixteen inoculations was made. The nasal exudate from diseased horses was suspended in salt solution and extracts of the pneumonic lung were prepared and both filtered. In no instance was influenza produced by the injection of filtrates. Intranasal sprays with the sediment obtained from 180 cc. of an ascitic dextrose broth culture produced a severe rhinitis

and pleuritis accompanied with fever and rapid pulse and respiration. The horse recovered in 18 days. Another horse was injected intravenously with sediment from the same amount of culture. It developed symptoms of influenza and died 8 days later. Autopsy revealed fibrinous pleuritis, bronchopneumonia, and evidences of septicemia.

Since the relationship of the strangles streptococcus with those associated with influenza seemed confused, it was considered advisable to study these streptococci with the methods now in use. In addition, the problem of ascertaining whether streptococci similar to those found in strangles and influenza were carried on the nasal mucosa or in the pharynx of horses presented itself. Studies were made on the following classes of horses: (1) normal horses (eastern) that had been in this vicinity for some time; (2) apparently normal horses that had recently been shipped from the West; (3) those suffering from strangles, influenza, rhinitis, and purulent conditions.

Streptococci of the Nasal Mucosa and Upper Pharynx of Normal Horses.

Sterile cotton swabs wound on bale wire, 15 and 30 cm. in length, were employed to obtain material. The shorter were used for the nasal passages and the longer for the pharynx. The same swab was used in both nostrils. In making preparations from the throat the mouth was held open with a speculum, the tongue depressed, and the swab brushed over the mucosa of the upper pharynx. Within 2 hours the swabs were agitated for a few seconds in tubes containing 10 cc. of sterile 0.9 per cent salt solution. Two or three loopfuls of suspension were inoculated into a tube containing 12 cc. of melted veal infusion agar (2 per cent) cooled to 45°C. and the whole was plated with 1 cc. of defibrinated horse blood. After 24 hours incubation at 38°C. subcultures were made from colonies resembling streptococci.

The results of the examinations of the thirty eastern horses may be summarized as follows:

Horses carrying non-hemolytic streptococci on the nasal mucosa	8
Horses carrying hemolytic streptococci on the nasal mucosa	0
Horses carrying non-hemolytic streptococci in the pharynx	6
Horses carrying hemolytic streptococci in the pharynx	18

The morphological and cultural characters of these streptococci are given in Tables I, II, and III. Inoculations from 18 hour broth cultures were made into tubes containing 13 cc. of fermented bouillon adjusted to +0.6 to +0.8 (phenolphthalein), to which had been added sufficient amounts of the carbohydrates and other substances to make a 1 per cent solution. Titrations were made after 5 days incubation at 38°C. An incubation period of this length was considered sufficient since the maximum acid production is usually reached within 36 hours. The amounts recorded in the tables represent the net acid production.¹ Litmus milk was likewise incubated for 5 days. On removal from the incubator if not coagulated the tubes were boiled for a few minutes. The figures under the column "Diameter of area of hemolysis" represent the diameter of hemolysis about the deep colonies after 24 hours incubation.

Smith and Brown (19) and Brown (20) describe two principal types of hemolysis. The alpha type is characterized by a partial hemolysis and greenish discoloration of the red cells immediately surrounding the deep colonies. This zone is succeeded by another clear band of partially hemolyzed cells. In the beta type the hemolytic area is sharply defined and clear, since the cells are completely hemolyzed. The beta type has been frequently observed among the horse streptococci but the alpha type has not been met with. A characteristic hemolytic zone has been noted with considerable frequency in the throat strains from horses. It surrounds both the surface and deep colonies as a clearly defined area varying in color from light orange to a delicate red. In certain instances there is a narrow clearer line between the colony and the larger discolored area. Microscopic examination reveals the presence of aggregates of unhemolyzed red cells throughout the lighter colored zone. The intensity of color appears to depend on the number of unhemolyzed cells. If the incubated plates are stored for 24 or 48 hours the color of the unhemolyzed cells has a tendency to deepen. When rabbit blood is used in the plate cultures the picture is not so striking. Colonies surrounded by the narrow clear line have been indicated

¹ The total acidity reached in the various test substances may be approximated by adding 0.7 per cent to the amounts given in the table.

in the tables as Type xa (Figs. 1 and 2), and those which showed only the partially hemolyzed area as Type x (Figs. 3 and 4).

Since all strains were Gram-positive, mention of this characteristic has been omitted from the tables.

The nasal strains fall into two groups when classified on the basis of lactose fermentation. In both the proportion of mannite-fermenting strains is high. The predominating type is a streptococcus which produces acid in dextrose, lactose, saccharose, maltose, man-

TABLE I.

Non-Hemolytic Streptococci from the Nasal Mucosa of Eastern Horses.

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
N 5	L. C.*	Clear.	Coagulated on boiling.	3.0	2.6	2.4	2.4	0.0	0.0	2.6	1.9
N 8	"	"	" " "	3.4	2.8	2.8	2.5	0.0	0.0	2.5	2.6
N 9	"	Turbid.	" " "	2.7	2.8	2.8	2.4	0.0	0.0	1.9	2.1
N 10	"	"	" " "	2.5	2.3	2.4	2.2	0.1	0.2	1.9	2.4
N 11	"	Clear.	" " "	2.3	2.1	0.0	2.2	0.0	0.0	1.7	2.3
N 13	"	"	" " "	2.5	2.4	2.3	2.4	0.2	0.0	2.7	2.0
N 15	"	"	" " "	3.2	3.5	2.2	3.0	0.2	0.1	2.7	2.5
N 19	"	"	" " "	3.5	3.5	3.5	3.0	0.0	0.0	2.5	2.3
N 6	Pairs and S. C.	Turbid.	Unchanged.	1.7	0.2	1.4	1.5	0.2	0.2	0.2	1.6
N 16	" " "	"	"	1.8	0.0	1.8	1.8	0.1	0.0	0.0	1.9
N 3	L. C.	Clear.	"	3.0	0.1	2.7	3.0	2.8	0.1	2.7	3.2
N 4	"	"	"	2.4	0.1	2.4	2.4	0.1	0.1	2.5	2.4

* L. C. indicates chains of 20 or more elements; threads of 8 to 20 are indicated as M. C., and those made up of 6 or 8 cocci as S. C.

nite, and salicin. One strain failed to ferment saccharose. None of these streptococci proved pathogenic for mice when 0.1 cc. of a 24 hour bouillon culture was injected into the peritoneal cavity.

Six of the seven strains of non-hemolytic streptococci from the pharynx failed to ferment lactose. They differed from the nasal types in their inability to ferment mannite. All except one attacked raffinose or inulin. The hemolytic streptococci possessed similar fermentative characters, although the proportion of lactose-fermenting

and non-lactose-fermenting strains was nearly equal. They too were capable of breaking down raffinose or inulin. Acid production in mannite was not infrequent but always occurred in combination with raffinose or inulin fermentation. Strain P 14 differed sharply from the others in its cultural characters and its pathogenic properties. This species was extremely virulent for mice and rabbits. The others failed to produce ill effects when 0.1 cc. of a 24 hour bouillon culture was injected into the peritoneal cavity of mice, or when 1 cc. was injected intravenously into rabbits.

TABLE II.

Non-Hemolytic Streptococci from the Pharynx of Eastern Horses.

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
P 41	Pairs and S. C.	Turbid.	Unchanged.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
P 45	L. C.	"	"	4.3	0.0	3.7	4.0	3.5	3.4	0.0	3.1
P 13	Pairs.	Clear.	"	3.0	0.1	2.4	2.6	2.5	3.0	0.2	3.0
P 42	L. C.	Turbid.	"	3.8	0.1	2.6	3.0	0.3	2.6	0.1	2.7
P 52	"	"	"	4.8	0.0	4.4	4.5	0.0	4.7	0.1	4.8
P 5	M. C.	"	"	3.2	0.1	3.0	2.9	0.1	2.7	0.1	2.8
P 36	"	"	Coagulated on boiling.	4.7	0.1	4.5	3.2	0.1	0.2	0.1	3.9
				3.4	2.9	3.5	3.6	2.5	3.6	0.1	3.5

From these studies several points stand out clearly. The bulk of the nasal flora is made up of non-hemolytic streptococci which ferment mannite. In the pharynx both hemolytic and non-hemolytic streptococci which ferment raffinose and inulin predominate. Strains which fail to produce acid in lactose are frequent in both regions.

Since the preceding results had established sufficiently the streptococcic flora of normal horses which had been in the East for some time, studies were begun on horses which had been recently shipped from the West. These animals were apparently normal when the cultures were made. Horses were examined over a period of 5 months. It may be assumed that they had been exposed to influenza and other diseases at various shipping points. Some of their fellows were

TABLE III.

Hemolytic Streptococci from the Pharynx of Eastern Horses.

Strain No.	Group- ing.	Growth in bouillon.	Litmus milk.	Production of acid in.								Diameter of area of hemol- ysis.	Type of hemol- ysis.
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.		
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mm.	
P 3	L. C.	Clear.	Unchanged.	2.1	0.0	1.9	2.0	1.7	1.9	0.0	2.0	3.0-3.5	Beta.
P 16	"	"	"	2.7	0.0	2.8	2.8	2.4	2.8	0.0	3.3	4.0-5.0	x
P 19	"	Turbid.	"	2.8	0.1	2.5	2.8	2.3	3.2	0.0	2.8	3.0	x
P 20	"	Clear.	"	1.3	0.0	1.4	1.5	1.9	1.3	0.0	1.8	3.5-4.0	Beta.
P 30	"	"	"	2.8	0.0	2.6	2.6	2.2	1.7	0.0	2.2	1.0	x
P 39	M. C.	"	"	2.8	0.0	2.2	2.1	2.2	1.8	0.0	0.6	2.5-3.0	x
P 43	Pairs and S. C.	Turbid.	"	4.0	0.0	3.7	4.0	3.5	3.4	0.0	0.8	3.5-4.0	x
P 11	" " "	"	"	3.7	0.3	3.7	3.5	2.9	1.7	3.3	3.3	2.0-2.5	x
P 24	M. C.	"	"	2.6	0.0	2.3	2.6	0.0	2.2	0.0	2.4	2.5-3.0	Beta.
P 47	Pairs and S. C.	Clear.	"	2.4	0.0	2.4	2.2	0.0	2.4	0.0	2.4	2.5-3.0	x
P 26	S. C.	Turbid.	"	4.6	0.0	4.2	4.0	0.0	4.3	2.2	4.2	2.0	x
P 22	L. C.	Clear.	Coagulated on boiling.	1.7	1.3	1.7	1.8	1.4	1.8	0.0	1.8	3.0-4.0	x
P 35	"	"	" " "	3.8	3.5	3.8	3.5	0.6	3.7	0.2	4.0	3.0-3.5	x
P 38	M. C.	"	" " "	2.9	2.7	2.9	2.7	2.5	2.1	0.0	3.0	2.5-3.0	Beta.
P 51	L. C.	"	" " "	3.6	2.1	3.4	3.1	2.7	2.9	0.0	2.6	4.5-5.0	x
P 23	Pairs and S. C.	Turbid.	Acid.	2.8	2.6	3.0	3.1	2.5	2.3	2.8	2.9	2.5-3.0	Beta.
P 1	Pairs.	"	Coagulated on boiling.	3.4	3.1	3.2	3.3	2.9	0.0	3.1	2.9	4.0-5.0	xa
P 6	" and S. C.	"	" " "	3.1	2.7	3.1	3.2	2.6	0.0	2.6	2.9	4.0-5.0	xa
P 53	" " "	"	Acid.	3.8	3.2	3.7	3.8	3.2	0.0	2.9	3.8	3.0-3.5	x
P 40	M. C.	"	Firmly coag- ulated.	4.7	4.5	5.2	5.2	4.2	0.2	0.0	4.1	6.0-7.0	xa
P 14	"	Clear.	Coagulated on boiling.	3.4	2.8	3.1	3.3	0.0	0.0	0.0	2.4	3.0-3.5	Beta.

suffering from influenza when the cultures were made. In all probability many had been exposed repeatedly. It was impossible to observe them after they had left the sales stables; some may have come down later.

The following summarizes the results of the nasal and pharyngeal examinations of the twenty-three western horses:

Horses carrying non-hemolytic streptococci on the nasal mucosa	8
Horses carrying hemolytic streptococci on the nasal mucosa	8
Horses carrying non-hemolytic streptococci in the pharynx	11
Horses carrying hemolytic streptococci in the pharynx	11

TABLE IV.

Non-Hemolytic Streptococci from the Nasal Mucosa of Western Horses.

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Rafinose.	Inulin.	Mannite.	Salicin.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
N 26	L. C.	Turbid.	Coagulated, not firmly.	4.1	3.0	3.9	3.6	0.0	0.0	2.7	3.5
N 40	"	Clear.	" " on boiling.	3.5	3.7	3.7	3.3	0.0	0.2	2.7	2.6
N 44	"	"	" " "	2.9	2.5	3.4	3.1	0.0	0.0	2.9	2.5
N 45	"	"	" " "	3.9	3.2	3.0	3.4	0.1	0.1	3.0	4.1
N 27	Pairs and S. C.	Turbid.	" " "	3.2	3.0	3.1	3.2	0.0	3.2	0.0	0.0
N 41	" " "	"	" " "	3.6	3.4	3.8	3.8	0.1	3.3	0.1	0.0
N 24	M. C.	"	" " "	3.7	3.1	3.7	3.5	0.0	0.0	0.0	3.0
N 33	"	Clear.	Unchanged.	3.3	0.1	3.4	3.4	2.7	3.1	0.0	3.2
N 46	"	"	"	4.2	0.1	3.9	3.6	3.4	0.0	3.0	5.0

An outstanding feature is the presence of hemolytic streptococci in the lower nasal passages of 30 per cent of the western horses. Streptococci of this type were not found in the same region in the eastern horses. The morphological and cultural characters are given in Tables IV to VII.

It is interesting to observe that the non-hemolytic streptococci from the nasal mucosa are similar in both eastern and western horses. Mannite fermenters make up the majority of the flora, although raffinose and inulin fermentation was observed more frequently. A striking difference in the flora of the two classes of horses is the pres-

TABLE V.

Hemolytic Streptococci from the Nasal Mucosa of Western Horses.

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Diameter of area of hemolysis.	Type of hemolysis.
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.		
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mm.	
N 20	M. C.	Turbid.	Coagulated on boiling.	3.8	3.0	3.6	3.6	0.0	0.0	0.0	3.4	3.5-4.0	Beta.
N 25	L. C.	"	" " "	4.2	4.0	4.4	4.3	0.1	0.0	0.0	3.4	3.0-3.5	"
N 28	"	"	" " "	4.3	3.8	4.5	4.2	0.0	0.2	0.0	3.0	2.5-3.0	"
N 29	"	Clear.	" " "	4.6	3.2	4.2	4.2	0.0	0.0	0.0	3.4	3.5-4.0	"
N 38	M. C.	Turbid.	" " "	4.2	3.7	3.8	4.7	0.0	0.1	0.1	3.8	3.5-4.0	"
N 42	"	"	" " "	4.8	3.8	4.0	3.8	0.0	0.0	0.0	4.4	3.0	"
N 47	S. C.	Clear.	" " "	4.2	3.6	4.1	4.1	0.0	0.0	0.0	3.6	3.5-4.0	"
N 48	M. C.	"	" " "	4.1	3.6	4.0	4.0	0.2	0.2	0.0	3.5	3.5-4.0	"

TABLE VI.

Non-Hemolytic Streptococci from the Pharynx of Western Horses.

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
P 48	L. C.	Turbid.	Unchanged.	3.5	0.0	3.0	3.3	3.0	3.2	0.2	3.1
P 67	S. C.	"	"	2.7	0.1	2.6	2.7	3.1	4.0	0.0	3.6
P 73	L. C.	"	"	3.9	0.0	3.8	4.0	3.2	3.4	0.0	3.3
P 74	"	"	"	4.2	0.0	3.7	3.8	3.5	4.3	0.1	3.9
P 84	S. C.	"	"	4.0	0.0	4.2	4.2	3.5	3.3	4.0	4.0
P 64	L. C.	Clear.	"	7.2	0.0	6.4	5.7	5.6	0.0	0.0	5.4
P 75	"	Turbid.	"	3.8	0.0	4.0	4.1	0.0	3.7	0.0	3.5
P 76	M. C.	"	"	3.3	0.2	3.4	3.1	0.0	3.3	0.0	2.7
P 58	Pairs and S. C.	"	Firmly coagulated.	4.4	3.6	3.5	3.4	3.3	2.9	0.0	3.0
P 60	L. C.	"	Coagulated on boiling.	3.6	3.0	3.6	3.3	3.5	3.7	0.0	3.6
P 69	M. C.	"	" " "	2.9	2.3	3.3	2.9	2.2	2.8	0.0	1.7
P 72	L. C.	"	" " "	3.8	3.0	3.9	4.3	3.4	3.2	0.0	3.7
P 78	"	Clear.	" not firmly.	4.1	3.5	3.1	3.8	2.9	4.1	0.0	3.2
P 83	S. C.	Turbid.	" on boiling.	4.1	3.2	4.0	3.9	3.4	3.8	0.1	3.6

ence of pathogenic hemolytic streptococci (*Streptococcus pyogenes*) on the nasal mucosa of the western horses (Table V). These streptococci produced acid in dextrose, lactose, saccharose, maltose, salicin, and milk. The other substances were not fermented. Mice, when injected intraperitoneally with 0.1 cc. of a 24 hour bouillon culture, developed septicemia and died within 24 or 48 hours. Rabbits when injected intravenously with 1 cc. of culture were much more resistant;

TABLE VII.

Hemolytic Streptococci from the Pharynx of Western Horses.

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Diameter of area of hemolysis.	Type of hemolysis.
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.		
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mm.	
P 55	L. C.	Clear.	Coagulated on boiling.	4.0	3.3	3.9	3.6	0.1	0.0	0.0	4.0	3.0-3.5	Beta.
P 56	S. C.	"	Acid.	3.8	3.4	4.0	4.0	0.0	0.0	0.0	3.4	3.0-3.5	"
P 61	L. C.	Turbid.	Coagulated on boiling.	4.2	3.3	4.0	4.4	0.0	0.1	0.0	4.2	2.5-3.0	"
P 62	"	Clear.	" " "	4.4	3.6	4.1	4.5	0.1	0.2	0.0	3.6	3.0-3.5	"
P 63	M. C.	"	" " "	4.2	3.0	3.9	4.3	0.0	0.0	0.0	4.1	3.0-3.5	"
P 79	L. C.	Turbid.	Coagulated, not firmly.	4.6	3.9	4.3	4.5	0.1	0.0	0.0	5.0	3.0-3.5	"
P 80	S. C.	Clear.	" " "	5.3	4.3	4.9	5.3	0.0	0.0	0.0	4.6	3.5-4.0	"
P 81	L. C.	"	" " "	5.3	3.9	5.1	5.3	0.0	0.1	0.1	4.9	3.5-4.0	"
P 57	S. C.	Turbid.	Unchanged.	3.9	0.0	3.9	4.0	3.3	3.3	3.6	3.7	3.5-4.0	x
P 68	"	"	"	3.8	0.0	4.0	4.2	3.0	2.8	2.9	3.7	6.0-7.0	xa
P 66	L. C.	Clear.	"	2.0	0.0	1.8	1.9	2.4	2.0	0.0	1.7	2.5-3.0	xa
P 82	M. C.	"	Coagulated on boiling.	5.0	3.9	4.2	4.4	0.0	5.5	2.6	3.7	3.0-3.5	Beta.
P 65	L. C.	Turbid.	" " "	5.2	3.8	6.0	5.2	4.0	0.0	0.0	4.1	3.5-4.0	"

many became emaciated and showed severe temperature reactions. Some recovered while others developed purulent arthritis. Larger doses of culture usually produced septicemia.

The same groups which were encountered in the pharyngeal flora of eastern horses were observed in the western horses. Among the non-hemolytic streptococci raffinose- and inulin-fermenting types predominated. Strains which failed to ferment lactose were frequent.

The horses from the West carried *Streptococcus pyogenes* in the throat in eight instances. The other five strains given in Table VII are raffinose or inulin fermenters which may or may not act upon mannite. They are similar to those observed in Table III. Three streptococci of the *pyogenes* type from the pharynx possessed the same pathogenic properties for rabbits and mice as those found on the nasal mucosa.

Pathogenic Streptococci of the Nasal Mucosa and Upper Pharynx of Diseased Horses.

Pathogenic streptococci have been isolated from horses suffering from acute diseases of the respiratory tract (influenza, rhinitis, pharyngitis, and strangles) and from infected wounds. The material was obtained by brushing the affected surfaces with a sterile swab. Salt solution suspensions were then made. Plates were prepared within a few hours.

Among twenty-four cases of influenza hemolytic streptococci were found in considerable numbers in the nasal discharge from twenty-two. The plate cultures from the pharynx were positive in eight cases. When conjunctivitis (pink eye) was an accompanying factor streptococci were obtained in practically pure culture from the ocular exudate. It was possible to obtain material from six cases of strangles. Pus from various acute and chronic purulent conditions was suspended in salt solution from which plate cultures were made. Streptococci were frequently found in pure culture. Table VIII indicates the cultural characteristics of these streptococci.

It will be observed that the streptococci fall into two broad groups. The larger group, composed of streptococci from influenza, strangles (three cases), and all but one abscess are of *Streptococcus pyogenes* type. They produce acid in dextrose, lactose, saccharose, maltose, and milk but fail to acidulate raffinose, inulin, or mannite. The smaller group, *Streptococcus equi*, includes the non-lactose-fermenting strains which do not produce acid in raffinose, inulin, mannite, or milk. These were found in influenza and strangles, rhinitis and pharyngitis, and once in an abscess.

Both types are especially virulent for mice. Rabbits are more resistant. 1 cc. of a 24 hour bouillon culture of certain strains may

TABLE VIII.
Pathogenic Streptococci.

Strain No.	Source.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Diameter of area of hemolysis.
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.	
In. 14	Nasal exudate; influenza.	L. C.	Clear.	Coagulated on boiling.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mm.
In. 15	"	"	"	"	3.5	2.8	3.5	3.6	0.0	0.0	0.0	0.3	5.4
In. 19	"	"	Turbid.	"	3.7	2.9	3.8	3.6	0.0	1.0	0.3	7.3	0.3
In. 20	"	Pairs and S. C.	Clear.	"	4.2	3.3	3.9	4.4	1.0	0.0	0.4	3.3	0.3
In. 22	Pharynx;	L. C.	"	"	4.0	3.4	4.2	4.1	0.0	0.0	0.3	9.4	5.0
In. 25	Nasal exudate;	"	"	"	3.9	3.7	3.5	3.7	0.0	0.0	0.3	9.3	5.4
In. 26	"	"	"	"	5.0	3.8	4.7	4.2	0.0	0.0	0.4	3.3	0.3
In. 31	"	"	Turbid.	"	4.1	3.0	4.0	3.8	0.0	0.0	0.3	5.3	0.3
In. 32	"	"	"	"	4.0	3.4	4.1	4.0	0.0	0.0	0.3	6.3	5.4
In. 36	Pharynx;	M. C.	"	"	5.0	4.1	4.7	5.0	0.0	0.0	1.4	1.3	5.4
In. 38	Nasal exudate;	S. C.	Clear.	"	4.3	3.5	3.9	4.2	0.0	0.0	0.3	4.3	0.3
In. 42	Exudate from eye;	M. C.	"	"	4.2	3.5	4.2	4.4	0.0	0.0	0.4	3.3	0.3
In. 43	"	"	Turbid.	"	4.2	3.1	4.1	4.4	0.0	0.0	0.3	3.2	5.3
In. 46	Nasal exudate;	"	Clear.	"	4.6	3.6	4.2	4.8	0.1	0.0	0.2	4.1	2.5
In. 47	"	"	"	"	4.1	3.1	4.0	4.0	0.0	1.0	0.3	4.2	5.3
In. 48	Pharynx;	Pairs and S. C.	Turbid.	"	4.3	3.7	4.1	4.0	0.0	0.0	0.3	4.2	5.3
In. 49	Nasal exudate;	M. C.	"	"	4.2	3.1	3.7	4.2	0.0	0.0	1.3	0.3	0.3
In. 50	"	S. C.	"	"	4.2	2.9	4.0	4.3	0.0	0.0	0.4	0.3	5.4
In. 52	Pharynx;	M. C.	"	"	4.3	2.9	3.7	4.3	0.0	0.0	0.4	0.3	5.4
In. 53	"	Pairs and S. C.	"	"	4.1	2.8	4.1	4.0	0.0	1.0	0.3	8.4	0.4
					4.3	3.3	3.8	4.3	0.1	0.0	0.3	9.3	0.3

In. 54	Nasal exudate; influenza.	M. C.	Turbid.	Coagulated on boiling.	4.1.3.13.64.2.0.0.0.10.0.3.4.3.5.4.0
In. 55	"	L. C.	Clear.	"	4.3.3.64.2.4.2.0.0.0.0.3.5.3.5.4.0
In. 57	"	Pairs and S. C.	Turbid.	"	4.4.3.0.4.1.4.0.0.10.0.4.0.3.0.3.5
In. 58	"	"	"	"	4.6.3.0.4.2.4.3.0.0.0.0.3.8.3.5.4.0
In. 59	"	"	"	"	4.2.3.4.1.4.3.0.0.0.0.4.0.3.0.3.5
In. 60	"	S. C.	Clear.	"	4.1.3.0.3.7.3.9.0.0.0.0.3.2.0.2.5
In. 62	"	M. C.	Turbid.	"	3.7.3.3.3.5.3.5.0.0.0.0.3.2.3.0.3.5
In. 63	"	"	"	"	3.7.2.9.3.7.3.4.0.0.0.0.3.2.3.0.3.5
In. 64	"	L. C.	"	Partially coagulated on boiling.	4.0.3.1.3.0.4.1.0.0.0.0.3.2.3.0.3.5
In. 66	"	"	"	Acid.	3.9.3.1.3.8.3.9.0.0.0.10.0.3.4.2.0.2.5
In. 69	Pharynx;	"	"	Coagulated on boiling.	4.0.3.7.4.1.3.5.0.0.0.0.3.6.2.0.2.5
In. 70	"	"	"	"	3.2.3.2.3.1.3.8.0.0.0.0.1.3.2.0.2.5
In. 71	Nasal exudate;	"	Clear.	"	4.0.3.3.4.1.4.0.0.0.10.0.3.7.2.0.2.5
In. 76	"	S. C.	Turbid.	"	3.2.3.0.3.0.3.1.0.0.10.1.2.9.2.5.3.0
In. 77	Pharynx;	M. C.	Clear.	"	4.1.3.6.3.3.3.9.0.1.0.1.3.4.3.0.3.5
In. 79	Nasal exudate;	"	"	"	4.4.3.6.4.1.3.8.0.1.0.2.0.1.3.0.2.5.3.0
In. 81	"	"	Turbid.	"	3.7.2.7.3.3.3.7.0.1.0.1.3.2.2.5.3.0
In. 84	"	L. C.	Clear.	"	3.7.2.9.3.6.3.6.0.0.10.0.2.9.3.5.4.0
In. 88	"	"	Turbid.	"	3.1.2.9.3.1.3.4.0.0.10.0.2.3.2.5.3.0
In. 90	"	M. C.	"	"	3.8.3.1.3.6.3.5.0.1.0.0.0.2.7.2.5.3.0
Str. 1	"	"	"	"	4.4.3.5.4.1.4.2.0.1.0.1.0.3.8.3.0.3.5
Str. 20	"	"	"	Acid.	3.6.3.2.3.4.3.8.0.0.0.0.3.5.3.0.3.5
Str. 22	Abscess;	L. C.	Clear.	"	3.7.2.5.3.3.3.5.0.0.0.0.3.4.3.0.3.5
HA 2	"	S. C.	"	Coagulated on boiling.	3.7.2.9.3.7.3.6.0.0.0.0.3.1.2.5.3.0
HA 3	"	M. C.	Turbid.	"	4.2.3.3.3.8.3.3.0.0.10.0.4.1.3.5.4.0
HA 5	"	"	"	"	4.5.3.5.3.8.4.3.0.0.0.0.3.5.3.0.3.5
HA 6	"	"	"	"	4.6.3.7.3.9.4.3.0.0.10.0.3.6.3.5.4.0
HA 7	"	S. C.	Clear.	"	4.3.3.3.3.6.4.1.0.0.0.0.3.9.3.5.4.0
HA 10	"	M. C.	Turbid.	"	4.9.3.7.4.6.4.4.0.10.0.0.3.5.2.0.5
HA 13	"	L. C.	Clear.	"	3.3.3.0.3.4.3.3.0.0.1.0.0.3.1.3.5.4.0

TABLE VIII—Concluded.

Strain No.	Source.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Diameter of area of hemolysis.
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Saltin.	
					per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mm.
HA 16	Placenta; abortion.	M. C.	Turbid.	Coagulated on boiling.	3.6	3.1	3.6	3.6	0.0	0.0	0.3	1.3	0-3.5
HA 17	Metritis.	"	"	"	4.2	3.7	3.9	0.0	0.0	0.3	3.0	3.5	
HA 19	Abscess.	"	"	"	3.9	3.1	2.7	3.9	0.1	0.0	0.2	8.2	5-3.0
N 23	Nasal exudate; rhinitis.	"	"	"	1.4	3.6	3.8	4.0	0.0	0.0	1.3	9.3	0-3.5
In. 2	" influenza.	S. C.	Clear.	Unchanged.	4.3	0.0	3.9	4.4	0.1	0.0	0.3	4.3	0-3.5
In. 3	"	"	Turbid.	"	4.5	0.1	4.1	4.3	0.1	0.1	0.0	3.3	5-4.0
In. 5	Pharynx;	M. C.	"	"	4.4	0.1	4.0	4.4	0.0	0.0	0.3	6.3	0-3.5
In. 6	"	"	"	"	4.4	0.1	3.9	4.3	0.0	0.0	0.3	3.3	0-3.5
In. 9	Nasal exudate;	S. C.	Clear.	"	4.3	0.0	4.4	4.5	0.2	0.2	0.4	1.5	2-0
In. 10	"	"	"	"	4.7	0.2	4.4	4.7	0.0	0.0	0.3	9.1	5-2.0
In. 11	Exudate from eye;	"	Turbid.	"	4.5	0.0	4.1	4.4	0.0	0.0	0.3	4.3	0-3.5
In. 12	"	"	Clear.	"	4.4	0.1	4.1	4.5	0.0	0.1	0.3	4.3	5-4.0
In. 73	"	"	Turbid.	"	4.8	0.0	4.5	4.7	0.0	0.0	1.4	2.2	0-2.5
In. 86	"	M. C.	"	"	4.1	0.0	3.6	4.6	0.0	1.0	0.3	1.3	5-4.0
Str. 6	Nasal exudate; strangles.	S. C.	"	"	4.2	0.0	4.0	3.8	0.0	0.0	0.3	9.2	0-2.5
Str. 9	"	M. C.	"	"	4.2	0.0	3.7	4.4	0.0	0.0	0.3	5.3	0-3.5
Str. 12	Abscess;	"	"	"	4.0	0.0	3.7	4.1	0.1	0.0	0.3	2.3	0-3.5
Str. 16	"	S. C.	"	"	3.9	0.1	3.1	3.7	0.1	0.2	0.3	6.3	5-4.0
Str. 18	"	M. C.	"	"	3.1	0.0	3.5	3.2	0.0	0.0	0.2	6.3	5-4.0
Str. 23	"	Pairs and S. C.	"	"	3.8	0.1	3.8	4.0	0.1	0.1	0.3	0.3	0-3.5
HA 8	"	M. C.	Clear.	"	3.4	0.1	3.4	3.5	0.0	0.0	0.3	5.2	0-2.5
N 23A	Nasal exudate; rhinitis.	"	Turbid.	"	4.6	0.1	4.6	4.6	0.1	0.1	0.4	6.3	0-3.5
P 70	Pharyngitis.	L. C.	"	"	4.8	0.0	4.3	4.8	0.1	0.1	0.0	3.4	3.0

cause septicemia but usually produces severe febrile disturbances or causes localization in joints. Arthritis is the most frequent result of intravenous injections.

The colonies of *Streptococcus pyogenes* are round, slightly raised, translucent, and rarely exceed 1.5 mm. in diameter. The hemolytic area is of the beta type and is pronounced about both the surface and deep colonies. The colonies of *Streptococcus equi* are larger, more opaque, sharply raised, and glistening. Hemolytic zones develop about both the surface and deep colonies. On horse blood agar slants the non-lactose-fermenting type produces a much thicker, glistening, opaque growth and resembles *Streptococcus mucosus*. The other forms a more delicate, thin dry film.

Preparations from the peritoneal exudate of mice dying as the result of inoculation, stained by Gram's method and counterstained with safranine, show both types grouped in pairs and short chains. A well developed capsular substance can usually be demonstrated about the cocci. In films made from the pus of infected horses *Streptococcus equi* usually appears as diplococci and chains of 10 or 12. In one instance extremely long chains were observed. *Streptococcus pyogenes* frequently occurs in pairs and in chains of 6 or 8.

DISCUSSION.

The true etiological relationship of streptococci to both strangles and influenza is still in doubt. There can be no question, however, that they play an important part in both diseases. *Streptococcus pyogenes* has been found more frequently in horses suffering from influenza than *Streptococcus equi*, although the latter species has been isolated from four typical cases. In strangles the opposite holds true, but in certain instances *Streptococcus pyogenes* has been isolated in pure culture. It is possible that nasal and pharyngeal infections depend, to a considerable degree, on a lowered resistance. Such a condition may exist either as the result of a distinct primary infection due to another organism or virus, or to a number of external causes. The proportion of infected animals following a journey appears to depend somewhat on atmospheric conditions, diminished rations, overcrowding, and the length of the journey. One fact which points to a cause or causes other than the streptococcus is the frequency

with which apparently normal horses carry virulent *Streptococcus pyogenes* on the nasal mucosa and in the throat. Such horses in this district are purchased by farmers who immediately house them with other horses on their farms. Influenza rarely develops among the resident horses. It is not uncommon, however, for an aged horse reared in the East to develop influenza or strangles after exposure to these diseases in sales stables.

It is becoming a common practice to attempt to immunize horses with killed cultures of streptococci before shipment. From this investigation it seems that these vaccines should contain both the lactose-fermenting *Streptococcus pyogenes* and the non-lactose-fermenting *Streptococcus equi*.

The horse streptococci, especially *Streptococcus pyogenes*, resemble those of human and bovine types to a certain extent. The cow strains, from mastitis, are easily separated from those of the horse by their low pathogenicity for mice and rabbits and their ability to coagulate milk. Bovine streptococci from mastitis rarely produce well marked areas of hemolysis about the surface colonies. Hemolytic zones usually develop about the surface colonies of the pathogenic human and equine strains. The pathogenic horse streptococci closely resemble those from human diseases in pathogenicity for rabbits and mice and in their hemolytic properties. Avery and Cullen (21) have shown that the hydrogen ion concentration of the bovine strains grown in dextrose broth ranges between 4.3 and 4.5, while that of the human strains is 5.0 to 5.2. These differences have held true when measured with 0.05 N sodium hydroxide. The acid production of the bovine strains from mastitis has averaged 4.4 per cent of normal acid, of those pathogenic for the horse the net production is a little lower, 4.1 per cent. The human strains, however, are considerably lower, 2.6 per cent. Thus acid production appears to serve as a ready means of differentiation between the pathogenic animal and human strains.

SUMMARY.

The lower nasal mucosa and the pharynx of thirty eastern and twenty-three western horses have been examined for streptococci.

Eight of the eastern horses carried non-hemolytic streptococci on the nasal mucosa. From the pharynx of six, non-hemolytic strepto-

cocci were cultivated. The throats of eighteen contained strains of the hemolytic type. The nasal mucosa of the eastern horses failed to show hemolytic streptococci.

Eight western horses carried non-hemolytic streptococci in the nasal passage; eight also harbored the hemolytic type. Twenty-two strains were isolated from the pharynx. Eleven were hemolytic.

Among all the non-hemolytic nasal strains those capable of fermenting mannite predominate. Those of the non-hemolytic types from the pharynx of both classes of horses may or may not ferment lactose but all do ferment either raffinose or inulin. In no instance have any of the non-hemolytic types proved pathogenic for mice.

The hemolytic strains from the nasal mucosa of the western horses were all of the *Streptococcus pyogenes* type. They were pathogenic for mice and rabbits. One strain from the pharynx of an eastern horse and eight from the throats of the western horses were of the same species. All the others corresponded closely in their fermentation reactions with non-hemolytic streptococci from the same region.

The streptococci from pathological sources were all hemolytic. They have fallen into two groups; the larger group (*Streptococcus pyogenes*) produced acid in dextrose, lactose, saccharose, maltose, milk, and salicin but failed to change the reaction of broth containing raffinose, inulin, or mannite. The streptococci of the smaller group (*Streptococcus equi*) differ only in their inability to ferment lactose or acidulate milk. Both types are pathogenic for mice. Rabbits are usually more resistant.

Streptococcus pyogenes has been isolated from eighteen of twenty-two cases of influenza, three of six cases of strangles, and from eight of nine abscesses. *Streptococcus equi* was observed in four horses suffering from influenza and five others affected with strangles. This species was also found in an abscess and associated with both rhinitis and pharyngitis.

I am indebted to Dr. W. J. Lentz of the Veterinary Department of the University of Pennsylvania for considerable material from abscesses, and to Dr. P. J. Runyon of Freehold, N. J., through whom a great portion of the material was obtained. Dr. R. B. Little of

this department assisted in collecting and also obtained much material.

BIBLIOGRAPHY.

1. Schütz, *Virchows Arch. path. Anat.*, 1887, cvii, 356.
2. Chantemesse and Delamotte, *Bull. Soc. anat. Paris*, 1888, lxiii, 694.
3. Schütz, *Arch. wissenschaft. u. prakt. Thierheilk.*, 1888, xiv, 172.
4. Baruchello, L., *Rev. gén. méd. vét.*, 1908, xi, 497.
5. Sand, G., and Jensen, C. O., *Deutsch. Z. Tiermed.*, 1888, xiii, 437.
6. Poels, J., *Fortschr. Med.*, 1888, vi, 4.
7. Galtier and Violet, *J. méd. vét. et zootech.*, 1889, xiv, series 3, 281.
8. Lignières, *Rec. méd. vét.*, 1897, iv, series 8, 437.
9. Ostertag, R., *Z. Infektionskrankh. Haustiere*, 1909, v, 180.
10. Pfeiler, W., *Z. Infektionskrankh. Haustiere*, 1910, viii, 155.
11. Todd, A. G., *J. Comp. Path. and Therap.*, 1910, xxii, 213.
12. Jensen, C. O., *Handbuch der Serumtherapie und Serumdiagnostik in der Veterinär-Medizin*, Leipsic, 1911, ii, 224.
13. Holth, cited by Jensen (12) and Bemelmans (14).
14. Bemelmans, E., *Centr. Bakteriöl., 1te Abt., Orig.*, 1913, lxx, 148.
15. Koch, J., and Pokschischewsky, N., *Z. Hyg. u. Infektionskrankh.*, 1913, lxxiv, 1.
16. Schofield, F. W., Second report on the investigation into joint-ill in foals existing in the Province of Ontario, Toronto, 1916.
17. Mathers, G., *J. Infect. Dis.*, 1918, xxii, 74.
18. Gaffky and Lührs, *Z. Veterinärk.*, 1913, xxv, 1.
19. Smith, T., and Brown, J. H., *J. Med. Research*, 1914-15, xxxi, 455.
20. Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, 1919.
21. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.

EXPLANATION OF PLATE 12.

FIG. 1. Plate Culture P 40. Hemolysis Type xa; 2 per cent veal infusion agar, 12 cc., defibrinated horse blood, 1 cc.; incubation 24 hours at 38°C. The inner clearer zone surrounded by the larger area of partial hemolysis is shown.

FIG. 2. A deep colony from the same culture. Note the aggregates of unhemolyzed cells in both zones. $\times 60$.

FIG. 3. Plate Culture P 57. Hemolysis Type x; media and incubation periods identical with those described under Fig. 1. The hemolytic area is more or less hazy due to a partial dissolution of the red cells.

FIG. 4. A deep colony of Culture P 57. Clumps of red cells are visible throughout the area of hemolysis. $\times 60$.

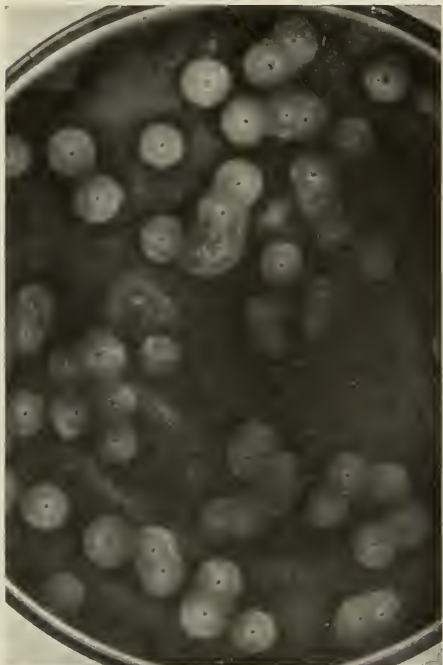


FIG. 1.

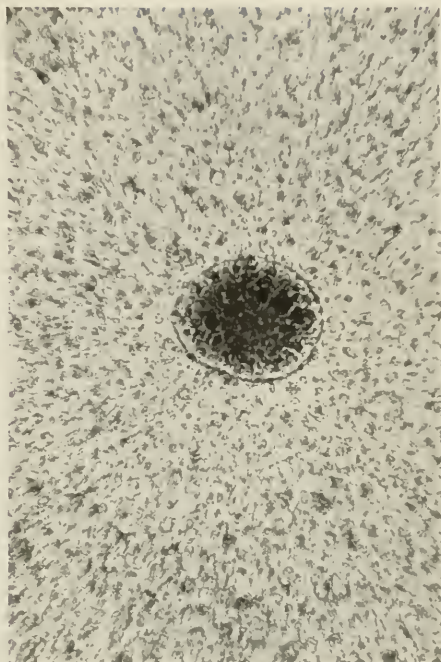


FIG. 2.

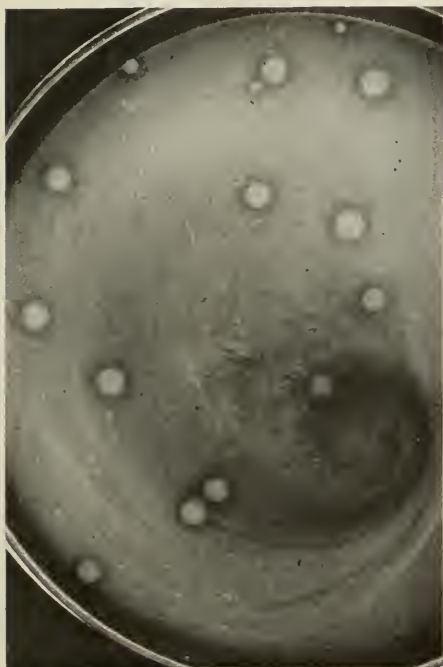


FIG. 3.

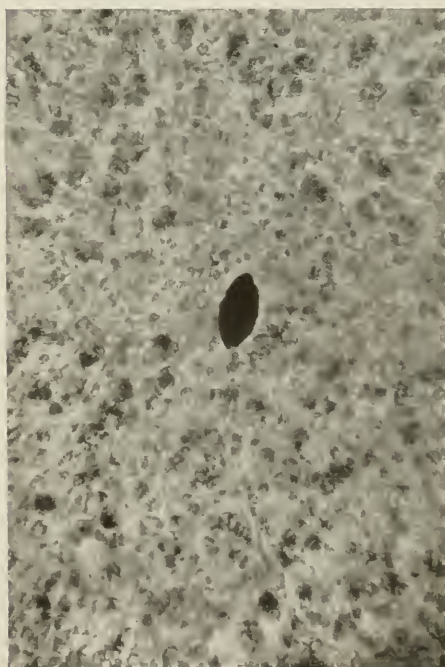
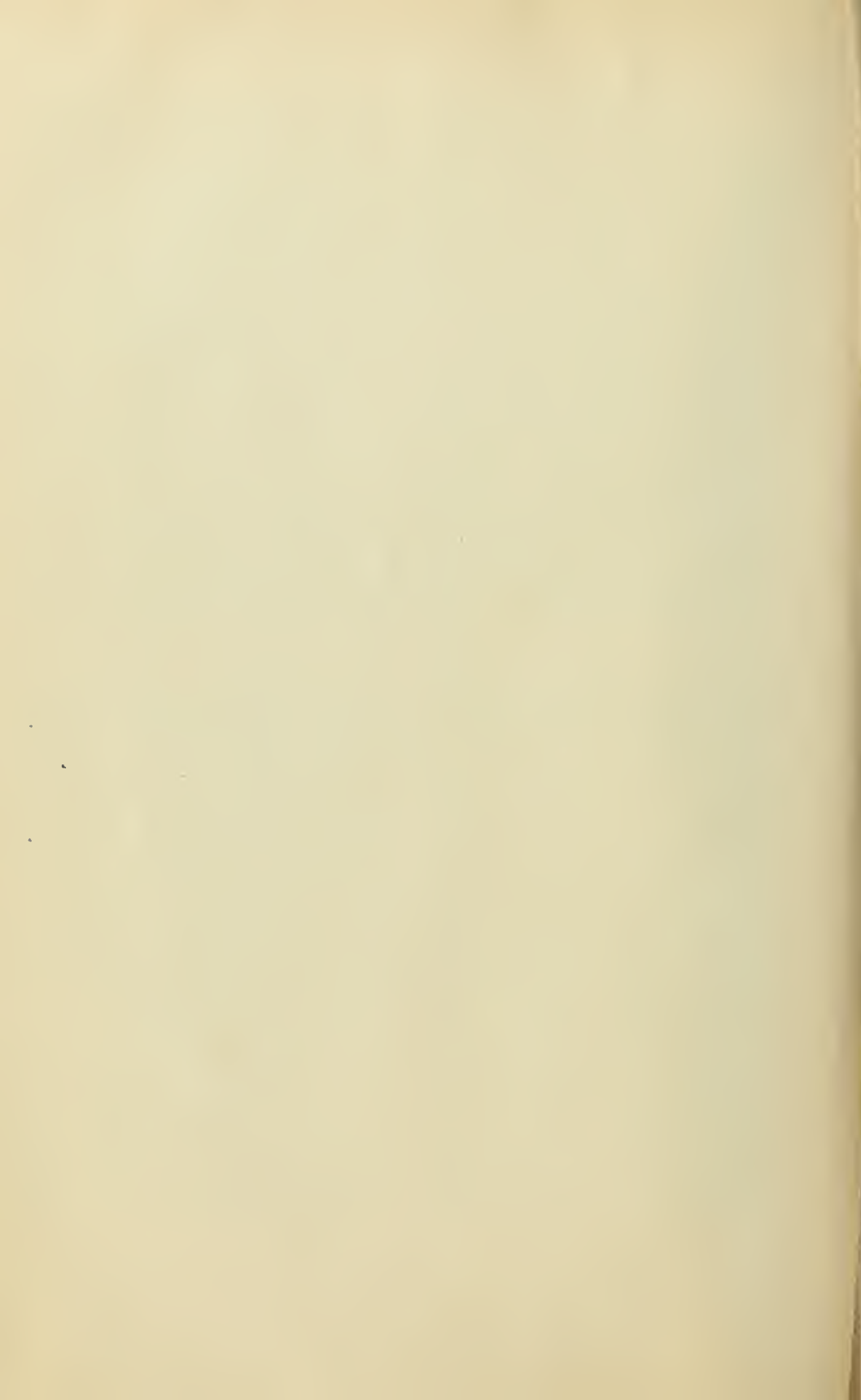


FIG. 4.

(Jones: Streptococci of equines.)



STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

I. ANTIGENIC RELATIONSHIPS BETWEEN STRAINS OF STREPTOCOCCUS HÆMOLYTICUS.

By A. R. DOCHEZ, M.D., O. T. AVERY, M.D., AND R. C. LANCEFIELD.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, June 1, 1919.)

The importance of the problem of the systematic classification of bacteria for the proper understanding and control of infectious diseases is becoming increasingly evident. Such study is necessary not only in the elucidation of the biological relationship existing between varieties of the same species of bacterium, but is also essential to the working out of epidemiological problems and to the development of knowledge useful in the effort to control infectious diseases by means of specific therapeutic and prophylactic measures. Bacteria closely resembling those responsible for the pathological process in many acute infections have been found to be present and to live, apparently without harm to the host, on the mucous membranes of a large proportion of normal individuals. The resemblance of the pathogenic to the harmless variety of microorganism is frequently so close that in many instances tests of particular specificity are required to show the existing biological differences. In fact, the problem in etiology today is to determine not only the bacterial species causing a given disease, but in addition the number of varieties of the same species that are pathogenic, and whether common and important non-pathogenic varieties exist. The study is one of varying complexity, and methods suitable to one species do not give the desired information when applied to another.

The purpose of such studies may be broadly defined as an effort to relate fixed and determinable characteristics of bacteria to pathogenicity. Though fluctuating variations of bacteria probably occur, it seems not unlikely that in most diseases a sufficiently constant equilibrium has been attained to justify the usefulness of the effort. The

methods employed in these studies are numerous. In some species morphological and cultural characters give important though somewhat limited information, and in many the biochemical reactions are of great significance. The most serviceable method, however, for obtaining the particular kind of knowledge desired is the study of immunological relationships. For some as yet unexplained reason the latter specific reactions are very constant among the pathogenic varieties. In the following study of *Streptococcus hæmolyticus* chief dependence has been placed on the knowledge obtained from the study of these immunity reactions.

In the various classifications of the streptococcus group as a whole that have been proposed, the custom has been to consider the strains that effect hemolysis of red blood cells as constituting a single or unit type (1). The validity of this assumption has been questioned and there has been much study and discussion of the probability of the existence of variations within this group, some evidence of which has been obtained from the study of sugar fermentations (2). Recent studies (3) indicate that certain broad lines of differentiation may be shown between hemolytic streptococci of human origin, and those of bovine origin whether found in milk or cheese. Hemolytic streptococci of the human type are usually found in association with some pathological process such as puerperal sepsis, septicemia, erysipelas, bronchopneumonia, or other conditions, and hemolytic activity is generally considered as an indication of pathogenicity. With the development of our knowledge (4), however, these organisms have been found with increasing frequency when no pathological lesion has been apparent. Investigators for many years have been interested in these strains, and discussion has centered about the unity or multiplicity of the group (5). The evidence in general favors the belief that hemolytic streptococci pathogenic for human beings comprise a single type. In the present paper it will be shown that by the use of properly controlled immune reactions differential characters between individual strains can be shown to exist.

During the winter of 1917-18 in the United States, there occurred in numerous localities a great increase in the incidence of a previously rather infrequent type of bronchopneumonia. The highest morbidity rate and earliest appearance of this disease were in military cantonments. In the spring of 1918, however, the type of infection under consideration was commonly observed in the civilian population. The disease first appeared as a secondary pneumonia following measles, but soon instances of apparently primary infection of the lungs were observed. Numerous studies of the bacteriology

of this condition have demonstrated that the infectious agent responsible for the lung lesion was in almost every instance *Streptococcus hæmolyticus*. As a result of the widespread incidence of the disease the latter organism became extensively distributed and was frequently found as a secondary invader in acute lobar pneumonia, and as a common inhabitant of the normal throat.

The material used in the present study was obtained from the military establishments in the neighborhood of Fort Sam Houston, Texas. The sources of the individual strains were the throats of patients suffering from acute measles, the sputum of patients with bronchopneumonia both primary and secondary to measles, pathological material obtained from cases of bronchopneumonia and acute lobar pneumonia, and the throats of healthy individuals who had been directly or indirectly exposed to infection in a variety of ways.

All the strains of hemolytic streptococcus employed in this study possess the typical characteristics of the group. They are hardy organisms and grow readily in meat infusion broth and on blood agar slants. They survive for many months when grown for 18 hours in rabbit blood broth and subsequently placed at refrigerator temperature. In meat infusion broth the growth has been of two types—a granular sediment with clear supernatant fluid and a flocculent sediment with turbidity throughout the remainder of the tube. All the organisms are strongly Gram-positive, grow in chains of varying length, and are bile-insoluble. Capsule formation has not been observed. On plates two types of colonies are seen—a small, round, smooth colony and a moist ameboid colony with a slightly roughened surface. All the strains are actively hemolytic, showing a wide zone of hemolysis on the surface of rabbit blood agar plates; hemolysis is complete in 2 hours, when a 5 per cent suspension of washed rabbit blood cells suspended in salt solution is mixed with an equal quantity of a 24 hour broth culture. The power of the different strains to ferment the usual test substances for streptococcus has been studied. The majority fall in the group of *Streptococcus pyogenes* according to Holman's classification of streptococcus on a basis of sugar fermentations. None of the strains ferments inulin and about 20 per cent of these actively hemolytic strains possess the power of fermenting mannite. The latter characteristic will be shown to have an inter-

esting relationship to the immunological classification of these organisms.

The virulence of this group of hemolytic streptococci is low for the ordinary laboratory animals in comparison with such an organism, for instance, as pneumococcus. Doses of 1 cc. or more of a 24 hour broth culture administered intraperitoneally are required to kill guinea pigs and rabbits. Furthermore, repeated passages through these animals fail to bring about a considerable accession of virulence. The fatal dose for white rats and mice is smaller, usually in the neighborhood of 0.1 cc. of a broth culture. By continuous passage through rats and mice it has been possible to raise the virulence of a certain number of strains to a point where 0.001 cc. of broth culture is lethal for a rat in 24 hours and 0.00000001 cc. for a white mouse. On the other hand, many strains cannot be raised to this high degree of virulence even after the most persevering effort. Once the maximum of virulence is attained, this quality persists without renewed animal passages for an indefinite period of time.

The sources of the strains of *Streptococcus hemolyticus* studied and some of their common characteristics are shown in Table I.

Although the finer differential classification of single species of bacteria by means of immune reactions is still in the earlier stages of development, enough evidence has been gathered to indicate that the more highly parasitic varieties of the species are more likely to consist of a limited number of unit types than are the less parasitic or the saprophytic members. In other words, unity of type seems to characterize the disease-producing microorganisms, whereas heterogeneity is more common among the non-pathogens. If this assumption is true it then becomes important to choose for purposes of classification the immune reactions which bring out most sharply the kind of differences sought, rather than a reaction which develops the basic relationship existing between all strains of the same species. For this purpose we regard the reactions of agglutination and protection as of superior usefulness to those of precipitation and complement fixation. The validity of any final classification arrived at depends, of course, upon the possibility of fitting accurately into such a classification a large number of strains freshly obtained from their natural environment.

TABLE I.

Source and Common Characters of Strains of Streptococcus hemolyticus Studied.

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
Type S 3.								
S 5	Autopsy (lung).	Bronchopneumonia following measles.	+++++	+	+	-	-	-
S 29	Sputum.	“ “	+++++	+	+	-	-	-
S 114	“	“ “	+++++	+	+	-	-	-
S 118	Pleural fluid.	“ “	+++++	+	+	-	-	-
S 124	Sputum.	“ “	+++++	+	+	-	-	-
S 145	Throat.	“ “	+++++	+	+	-	-	-
S 146	Chest fluid.	“ “	+++++	+	+	-	-	-
S 149	Blood.	“ “	+++++	+	+	-	-	-
S 151	Pleural fluid.	“ “	+++++	+	+	-	-	-
S 2	Autopsy (blood).	Bronchopneumonia.	+++++	+	+	-	-	-
S 3	“ (lung).	“	+++++	+	+	-	-	-
S 14	“ “	“	+++++	+	+	-	-	-
S 111	Chest fluid.	“	+++++	+	+	-	-	-
S 53	Throat.	Measles.	+++++	+	+	-	-	-
S 64	“	“	+++++	+	+	-	-	-
S 154	“	“	+++++	+	+	-	-	-
S 80	“	German measles.	+++++	+	+	-	-	-
S 140	“	“ “	+++++	+	+	-	-	-
S 11	Sputum.	Lobar pneumonia (Type I).	+++++	+	+	-	-	-
S 16	“	“ “	+++++	+	+	-	-	-
S 31	Throat.	“ “	+++++	+	+	-	-	-
S 44	“	“ “	+++++	+	+	-	-	-
S 83	“	“ “	+++++	+	+	-	-	-
S 95	“	“ “	+++++	+	+	-	-	-
S 125	“	“ “	+++++	+	+	-	-	-
S 144	“	“ “	+++++	+	+	-	-	-
S 8	“	Pneumonia.	+++++	+	+	-	-	-
S 131	“	“	+++++	+	+	-	-	-
S 41	“	Incipient tuberculosis.	+++++	+	+	-	-	-

TABLE I—Continued.

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
Type S 23.								
S 39	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	-	-	-
S 78	Pleural fluid.	“ “	++++	+	+	-	-	-
S 101	“ “	“ “	++++	+	+	-	-	-
S 107	Sputum.	“ “	++++	+	+	-	-	-
S 27	Autopsy (blood).	Bronchopneumonia.	++++	+	+	-	-	-
S 67	Blood.	“	++++	+	+	-	-	-
S 120	Autopsy (blood).	“	++++	+	+	-	-	-
S 98	Throat.	Measles.	++++	+	+	-	-	-
S 116	“	“	++++	+	+	-	-	-
S 117	“	“	++++	+	+	-	-	-
S 9	“	Lobar pneumonia.	++++	+	+	-	-	-
S 23	“	“ “	++++	+	+	-	-	-
S 56	Autopsy (lung).	“ “	++++	+	+	-	-	-
S 65	Sputum.	“ “	++++	+	+	-	-	-
S 75	Throat.	“ “	++++	+	+	-	-	-
S 133	“	“ “	++++	+	+	-	-	-
S 104	“	Pneumonia.	++++	+	+	-	-	-
S 130	“	“	++++	+	+	-	-	-
S 46	“	Incipient tuberculosis.	++++	+	+	-	-	-
S 122	“	“ “	++++	+	+	-	-	-

TABLE I—*Continued.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
Type S 60.								
S 137	Pleural fluid.	Bronchopneumonia following measles.	++++	+	+	+	—	—
S 6	Autopsy (lung).	Bronchopneumonia.	++++	+	+	+	—	—
S 35	Pleural fluid.	“	++++	+	+	+	—	—
S 55	Sputum.	“	++++	+	+	+	—	—
S 10	Throat.	Measles.	++++	+	+	+	—	—
S 37	“	“	++++	+	+	+	—	—
S 43	“	“	++++	+	+	+	—	—
S 60	“	“	++++	+	+	+	—	—
S 66	“	“	++++	+	+	+	—	—
S 71	“	“	++++	+	+	+	—	—
S 86	“	“	++++	+	+	+	—	—
S 88	“	“	++++	+	+	+	—	—
S 89	“	“	++++	+	+	+	—	—
S 100	“	“	++++	+	+	+	—	—
S 109	“	“	++++	+	+	+	—	—
S 123	“	“	++++	+	+	+	—	—
S 127	“	“	++++	+	+	+	—	—
S 128	“	“	++++	+	+	+	—	—
S 4	“	German measles.	++++	+	+	+	—	—
S 19	“	Lobar pneumonia.	++++	+	+	+	—	—
S 62	“	“ “	++++	+	+	+	—	—
S 72	“	“ “	++++	+	+	+	—	—
S 21	“	Incipient tuberculosis.	++++	+	+	+	—	—
S 141	“	“ “	++++	+	+	+	—	—
S 150	“	“ “	++++	+	+	+	—	—
S 267	Foot.	Cellulitis.	++++	+	+	+	—	—
S 269	Blood.	Erysipelas.	++++	+	+	+	—	—

TABLE I—*Continued.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
Type S 84.								
S 1	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	-	-	-
S 20	“ (blood).	“ “	++++	+	+	-	-	-
S 110	Sputum.	“ “	++++	+	+	-	-	-
S 138	“	“ “	++++	+	+	-	-	-
S 50	Pleural fluid.	Bronchopneumonia.	++++	+	+	-	-	-
S 84	“ “	“	++++	+	+	-	-	-
S 139	Throat.	“	++++	+	+	-	-	-
S 115	“	German measles.	++++	+	+	-	-	-
S 15	“	Lobar pneumonia.	++++	+	+	-	-	-
Unclassified.								
S 32	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	-	-	-
S 59	“ (blood).	“ “	++++	+	+	-	-	-
S 93	Sputum.	“ “	++++	+	+	-	-	-
S 97	Pleural fluid.	“ “	++++	+	+	-	-	-
S 136	Autopsy (lung).	“ “	++++	+	+	-	-	-
S 142	Sputum.	“ “	++++	+	+	-	-	-
S 49	“	Bronchopneumonia following German measles.	++++	+	+	-	-	-
S 24	Autopsy (lung).	Bronchopneumonia.	++++	+	+	-	-	-
S 18	Throat.	Measles.	++++	+	+	-	-	-
S 26	“	“	++++	+	+	-	-	-
S 42	“	“	++++	+	+	-	-	-
S 51	“	“	++	+	+	-	+	-
S 63	“	“	++++	=	+	-	-	-
S 96	“	“	++++	+	+	-	-	-
S 102	“	“	++++	+	+	-	-	-
S 106	“	“	++++	+	+	-	-	-
S 108	“	“	++++	=	+	-	-	-
S 148	“	“	++++	+	+	-	-	-

TABLE I—*Concluded.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
Unclassified— <i>Concluded.</i>								
S 17	Throat.	German measles.	+	—	—	+	—	—
S 47	"	" "	+++	+	—	—	—	—
S 48	"	" "	+++++	+	+	—	—	—
S 54	Sputum.	" "	+++++	+	+	—	—	—
S 68	Throat.	" "	+++++	+	+	—	—	—
S 69	Autopsy (lung).	Lobar pneumonia.	+++++	+	+	—	—	—
S 87	Sputum.	" "	+++++	+	+	—	—	—
S 90	Throat.	" "	+++++	+	+	—	—	—
S 99	Sputum.	" "	+++++	+	+	—	—	—
S 34	Throat.	Incipient tuberculosis.	+++++	+	+	+	—	+
S 36	"	" "	+++++	+	+	—	—	—
S 121	"	" "	+++++	+	—	—	—	—
S 129	"	" "	+++++	=	+	+	—	—
S 155	"	" "	+++++	+	+	—	—	—
S 264	Blood.	Osteomyelitis.	+++++	+	+	—	—	—
S 271	"	Septicemia.	+++++	+	+	—	—	—
S 272	Pus.	Abscess (measles).	+++++	+	+	—	—	—
S 273	"	Scarlet fever.	+++++	+	+	—	—	—
S 276	"	Pelvic abscess.	+++++	+	+	—	—	—
S 277	" (abdomen).	Peritonitis.	+++++	+	+	—	—	—
S 286	Pleural fluid.	Pneumonia.	+++++	+	+	—	—	—
S 288	Sputum.	"	+++++	+	+	—	—	—

The Reaction of Agglutination.

Specific agglutination has been found to be one of the most serviceable immune reactions for purposes of the biological classification of bacteria. In the typhoid and pneumococcus groups, for instance, it serves to distinguish clearly the different varieties from one another. It is likewise applicable to the classification of many other microorganisms. Efforts to classify the streptococci by means of this reaction apparently have not illuminated materially the relationship of one strain to another, nor have they shown a definite relationship between certain strains and a particular pathological process. A number of explanations of this fact may be proposed. In many instances no attention has been paid to the broader groupings of streptococci as determined by hemotoxin production and the fermentation of test sugars. Also streptococcus most frequently acts as a secondary invader in the production of disease, and it is probably an unwarranted assumption to suppose that type specificity is closely related to the character of the pathological process. One of the chief obstacles to the successful carrying out of the agglutination reaction has been the tendency of all types of streptococcus to undergo spontaneous granulation, and when used for tests to exhibit the phenomenon of non-specific agglutination. As a result of this, the reactions must usually be read against a more or less granular background, making it difficult, if not impossible, to distinguish between the non-specific and the specific influences. The tendency to spontaneous clumping is occasioned by several factors, only a few of which are understood. For instance, a homogeneous suspension of a granular streptococcus can easily be prepared by washing the organism several times with distilled water, and then resuspending in the same medium. The suspension will remain homogeneous for an indefinite period of time. If sodium chloride in concentrations above 0.06 per cent is added to the suspension, granulation immediately ensues. Many other salts act in the same manner. Substances antagonistic to this salt action may be added to the medium and function even to the extent of suspending the participation of the salt in the immune reaction, so that specific agglutination may be completely inhibited. Fortunately intermediate combinations can be found in which most

strains remain diffuse and in which the salt is still able to fulfill its part in the immune reaction. The most useful substance of this kind is ordinary meat infusion broth to which 1 per cent peptone has been added.¹ In addition, if streptococci are exposed to too great acidity the tendency to granulation is increased. In order to avoid this, the reaction of the medium may be so adjusted and such quantities of balanced phosphate solutions added that during growth an acidity greater than pH 7.1 is not attained. Certain other undetermined factors cause granulation, which may be defined as a general unsuitability of the medium for growth, and these can be eliminated only by experimenting with different preparations.

Technique.

The immune sera used in the agglutination and protection tests were obtained by the immunization of rabbits, sheep, and dogs. The animals were inoculated intravenously with repeated doses of heat-killed organisms, and in most instances a certain number of doses of living organisms was given. The employment for immunization of freshly isolated unpassed human strains, or the use of the same strains after a series of animal passages, does not alter in any recognizable way the specific qualities of the serum. The agglutinin and protective titer of the sera has remained undiminished for many months after the time of bleeding.

Great care is taken in the preparation of the organisms to be used in the agglutination reaction. The broth is made from carefully selected meat, and instead of the usual sodium chloride a sufficient quantity of a balanced phosphate mixture is added to give the required salt concentration and to adjust the hydrogen ion concentration at a pH of 7.4. When *Streptococcus hæmolyticus* is grown for 24 hours in a medium to which no sugar has been added, it does not develop an acidity greater than pH 7.2, which is just above the point at which the tendency to granulation appears. The organisms are removed from the culture medium by centrifugalization and are

¹ The authors are greatly indebted to Dr. Charles Krumwiede, Jr., of the Research Laboratories of the Department of Health of the City of New York, for many helpful suggestions in this technique.

TABLE III.

Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 3, with Strains of Streptococcus hemolyticus of Other Types and with Unclassified Strains.

Strain No.	Type of <i>S. hemolyticus</i> .	Serum.	Dilution.									
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 3	S 3	Type S 3	+	±	±	±	±	±	±	+	±	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 23	S 23	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 107	S 23	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 67	S 23	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 65	S 23	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 75	S 23	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 128	S 60	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 55	S 60	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 60	S 60	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 267	S 60	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 4	S 60	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 84	S 84	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 1	S 84	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 50	S 84	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 20	S 84	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 115	S 84	Type S 3	—	—	—	—	—	—	—	—	—	—
		Normal.	—	—	—	—	—	—	—	—	—	—
S 31	Unclassified.	Type S 3	+	+	+	+	+					—
		Normal.	—	—	—	—	—					—
S 108	“	Type S 3	—	—	—	—	—	—	—			—
		Normal.	—	—	—	—	—	—	—			—
S 87	“	Type S 3	—	—	—	—	—	—	—			—
		Normal.	—	—	—	—	—	—	—			—
S 288	“	Type S 3	—	—	—	—	—	—	—			—
		Normal.	—	—	—	—	—	—	—			—
S 42	“	Type S 3	—	—	—	—	—	—	—			—
		Normal.	—	—	—	—	—	—	—			—
S 121	“	Type S 3	+	±	±	—	—	—	—			—
		Normal.	—	—	—	—	—	—	—			—

allowed to continue longer, non-specific granulation occurs. If clumping develops in the broth controls or in more than the first two or three dilutions of normal serum, the reaction should be regarded as unsatisfactory and discarded. By the use of this technique, it has been possible to carry out reliable agglutination tests of various strains of *Streptococcus hæmolyticus* and to show that constant type rela-

TABLE IV.

Power of Antistreptococcus Serum, Type S 23, to Agglutinate Ten Representative Strains of the Same Type.

Strain No.	Serum.	Dilution.									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 23	Type S 23	++	++	++	++	++	++	+	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 107	Type S 23	++	++	++	++	++	++	++	+	±	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 27	Type S 23	+	++	++	++	++	+	±	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 39	Type S 23	+	+	++	++	++	+	±	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 56	Type S 23	+	+	++	++	++	+	±	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 67	Type S 23	+	+	++	++	+	+	±	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 98	Type S 23	++	++	++	++	++	++	++	+	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 101	Type S 23	++	++	++	++	++	++	++	+	±	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 104	Type S 23	+	++	++	++	++	++	++	+	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 130	Type S 23	++	++	++	++	++	++	+	+	+	-
	Normal.	-	-	-	-	-	-	-	-	-	-

tionships exist, and that the types are sharply differentiated from one another. The results of the application of the method to strains of streptococcus described above are shown in Tables II to IX.

In these tables are presented the agglutination reactions of a certain proportion of the total number of strains of *Streptococcus hæmolyticus* tested. An analysis of the results shows that in the collection of

TABLE V.

Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 23, with Strains of Streptococcus hemolyticus of Other Types and with Unclassified Strains.

Strain No.	Type of <i>S. hemolyticus</i> .	Serum.	Dilution.									
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 23	S 23	Type S 23	±	+	± ±	± ±	± ±	± ±	± ±	+	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 95	S 3	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 149	S 3	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 80	S 3	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 146	S 3	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 125	S 3	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 55	S 60	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 60	S 60	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 4	S 60	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 19	S 60	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 6	S 60	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 20	S 84	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 50	S 84	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 84	S 84	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 115	S 84	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 1	S 84	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 15	Unclassified.	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 116	"	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 49	"	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 69	"	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 155	"	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 18	"	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 99	"	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-
S 24	"	Type S 23	-	-	-	-	-	-	-	-	-	-
		Normal.	-	-	-	-	-	-	-	-	-	-

organisms studied it has been possible to detect four different types of *Streptococcus hæmolyticus*. These types have been noted as Types S 3, S 23, S 60, and S 84, from the number of the chosen representative. In addition to the type strains, there remains a residue of unclassified organisms. The summary for the total number of strains studied is given in Table X.

TABLE VI.

Power of Antistreptococcus Serum, Type S 60, to Agglutinate Ten Representative Strains of the Same Type.

Strain No.	Serum.	Dilution.									Broth.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
S 60	Type S 60 Normal.	++	++	++		++		++	++	+	-
S 269	Type S 60 Normal.	++	++	++	++	+	+	+	-		-
S 267	Type S 60 Normal.	++	++	++	++	++	++	+	+		-
S 55	Type S 60 Normal.	++	++	++		++		+	+	+	-
S 128	Type S 60 Normal.	++	++	++		++		++	++	+	-
S 72	Type S 60 Normal.	++	++		++		++	+	+	+	-
S 43	Type S 60 Normal.	++	++		++		++	++	++	+	-
S 123	Type S 60 Normal.	++	++		++		++	++	++	+	-
S 66	Type S 60 Normal.	++	++		++		++	++	+	+	-
S 127	Type S 60 Normal.	++	++		++		++	++	++	+	-

The total number of strains of *Streptococcus hæmolyticus* studied was 125. Of these, 85, or 68 per cent, are comprised in the types mentioned above, and 40, or 32 per cent, remain unclassified. Work with the unclassified strains is being continued and the indications are that a certain number of other types will be discovered. In fact, two new types have already been encountered, one comprising five

TABLE VII.

Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 60, with Strains of Streptococcus hæmolyticus of Other Types and with Unclassified Strains.

Strain No.	Type of <i>S. hæmolyticus</i> .	Serum.	Dilution.									Broth.
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
S 60	S 60	Type S 60	++	++	++	±	±	+	±	±		—
		Normal.	—	—	—	—	—	—	—	—		—
S 3	S 3	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 111	S 3	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 146	S 3	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 80	S 3	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 14	S 3	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 23	S 23	Type S 60	±	—	—	—	—	—	—	—		—
		Normal.	±	±	—	—	—	—	—	—		—
S 78	S 23	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 98	S 23	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 107	S 23	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 122	S 23	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 84	S 84	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 115	S 84	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 15	S 84	Type S 60	±	±	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 1	S 84	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 20	S 84	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 148	Unclassified.	Type S 60	+	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 96	"	Type S 60	+	±	±	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 286	"	Type S 60	±	±	—	±	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 47	"	Type S 60	±	+	+	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 102	"	Type S 60	—	+	+	+	±	±	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 48	"	Type S 60	±	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—
S 17	"	Type S 60	—	—	—	—	—	—	—	—		—
		Normal.	—	—	—	—	—	—	—	—		—

strains and another four strains, the immune reactions of which have not as yet been completed.

The antistreptococcus sera used in the agglutination reaction were obtained in the main by the immunization of sheep, a species of animal which yields a highly specific agglutinating serum for *Streptococcus hæmolyticus*. Agglutination occurred in all the type sera in dilutions of 1 : 1,000 or higher, with the exception of Type S 84, of which the

TABLE VIII.

Power of Antistreptococcus Serum, Type S 84, to Agglutinate Eight Representative Strains of the Same Type.

Strain No.	Serum.	Dilution.								
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120
S 84	Type S 84	+	±	+	±	+	±			
	Normal.	—		—	—	—	—			
S 1	Type S 84	+	±	+	±	+	±			
	Normal.	—	—	—	—	—	—			
S 50	Type S 84	+	±	+	±	+	±			
	Normal.	—	—	—	—	—	—			
S 20	Type S 84	+	+	+	±	±	—			
	Normal.	—	—	—	—	—	—			
S 15	Type S 84	+	±	±	±	+				
	Normal.	—	—	—	—	—				
S 115	Type S 84	+	±	±	±	±	—			
	Normal.	—	—	—	—	—				
S 139	Type S 84	+	±	±	±	+				
	Normal.	—	—	—	—	—				
S 138	Type S 84	+	±	±	±	+				
	Normal.	—	—	—	—	—				

agglutination titer has been consistently lower, usually not above 1:320. The agglutination titer of all the type sera for each strain of the same type has been approximately equal to the titer for the organism used for purposes of immunization. There has been strikingly little cross-agglutination between serum of one type and strains belonging to another. The same lack of crossing is observed among the unclassified strains with the few exceptions in which certain of these strains have shown some degree of agglutination in the type

TABLE IX.

Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 84, with Strains of Streptococcus hemolyticus of Other Types and with Unclassified Strains.

Strain No.	Type of <i>S. hemolyticus</i> .	Serum.	Dilution.									Broth.
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
S 84	S 84	Type S 84	++	++	++	+	+	+	+			-
		Normal.	-	-	-	-	-	-	-			
S 3	S 3	Type S 84	-	-	-	-	-	-	-			-
		Normal.	-	-	-	-	-	-	-			
S 14	S 3	Type S 84	-	-	-	-	-	-	-			-
		Normal.	-	-	-	-	-	-	-			
S 80	S 3	Type S 84	-	-	-	-	-	-	-			-
		Normal.	-	-	-	-	-	-	-			
S 146	S 3	Type S 84	-	-	-	-	-	-	-			-
		Normal.	-	-	-	-	-	-	-			
S 95	S 3	Type S 84	±	±	-	-	-	-	-			-
		Normal.	-	-	-	-	-	-	-			
S 23	S 23	Type S 84	±	±	-	-	-	-	-			-
		Normal.	±	±	-	-	-	-	-			
S 107	S 23	Type S 84	±	±	-	-	-	-	-			-
		Normal.	-	-	-	-	-	-	-			
S 122	S 23	Type S 84	-	-	-	-	-	-	-			-
		Normal.	-	-	-	-	-	-	-			
S 78	S 23	Type S 84	-	-	-	-	-	-	-			-
		Normal.	-	-	-	-	-	-	-			
S 98	S 23	Type S 84	±	-	-	-	-	-	-			-
		Normal.	±	±	-	-	-	-	-			
S 60	S 60	Type S 84	+	+	±	±	-	-	-			-
		Normal.	±	±	±	±	-	-	-			
S 128	S 60	Type S 84	++	+	+	+	-	-	-			-
		Normal.	+	+	±	±	-	-	-			
S 19	S 60	Type S 84	+	±	±	-	-	-	-			-
		Normal.	±	±	-	-	-	-	-			
S 141	S 60	Type S 84	+	±	±	-	-	-	-			-
		Normal.	±	±	±	-	-	-	-			
S 4	S 60	Type S 84	+	±	±	-	-	-	-			-
		Normal.	+	±	±	-	-	-	-			
S 108	Unclassified.	Type S 84	-	-	-	-	-					-
		Normal.	-	-	-	-	-					
S 97	"	Type S 84	-	-	-	-	-					-
		Normal.	-	-	-	-	-					
S 54	"	Type S 84	-	-	-	-	-					-
		Normal.	-	-	-	-	-					
S 34	"	Type S 84	-	-	-	-	-					-
		Normal.	-	-	-	-	-					
S 106	"	Type S 84	-	-	-	±	±					-
		Normal.	-	-	-	-	-					

sera, but not in sufficiently high dilutions to justify their inclusion within the types. These facts show that it is possible by a series of carefully conducted agglutination experiments to determine specific type relationships between strains of *Streptococcus hæmolyticus* and to show that the different types are immunologically distinct from one another. The clearness of the agglutination reactions presented is somewhat deceptive as to the ease and simplicity of the test. It must be remembered that *Streptococcus hæmolyticus* is notoriously

TABLE X.
Summary of Agglutination Reactions.

Type of <i>S. hæmolyticus</i> .	No. of strains.	Per cent.
S 3.....	29	23.2
S 23.....	20	16.0
S 60.....	27	21.6
S 84.....	9	7.2
Unclassified.....	40	32.0
Total typed.....	85	68.0
“ strains studied.....	125	

variable in its reactions, and that very slight and indeterminable changes in technique frequently obliterate almost completely the specificity of the agglutination reaction. In addition, a considerable number of strains is invincibly granular under all conditions and cannot be used, and occasionally strains are encountered which may occupy intermediate positions, the exact understanding of which needs a technique for the conduction of absorption experiments.

The Reaction of Protection.

Study of the power of antistreptococcus serum to protect animals against experimental infection with this organism has given rise to a number of different points of view, both regarding its action against strains from different sources, and against the same strain before and after animal passage, and also concerning the kind and the different

effect of varying antigens used in the process of immunization. For a full discussion of these matters the reader is referred to the general articles on streptococcus and to the more important papers dealing with these particular points (6). In this work they will only be considered where they have a particular bearing upon the subject under investigation. Although in the present paper the classification of *Streptococcus hemolyticus* by means of the agglutination reaction has been presented first, practically we have obtained our primary indications of the degree of antigenic differences between strains by means of the reaction of protection. Later each reaction has been used to confirm the information obtained by means of the other.

In the successful carrying out of protection experiments two points are of especial importance: first, the production of a serum of high potency; and second, the possibility of raising the virulence of the test strains of streptococcus to such a point that very minute doses of culture are sufficient to kill white mice in a limited period of time. We have been able to produce sera in the manner alluded to above of such potency that 0.5 cc. administered intraperitoneally is sufficient to protect a white mouse against 100,000 lethal doses of a highly virulent streptococcus. In order to produce such a serum many animals must be used, only a few of which may give the desired result. It has been possible to raise the virulence of many strains by continuous passage through white mice and rats to such a point that doses of from 0.000001 to 0.00000001 cc. of broth culture are sufficient to kill the former animals in from 24 to 48 hours. These seemingly difficult conditions must be attained in order that sufficiently long-range protective titers may be carried out to insure the reliability of the information obtained. Protection against one or two lethal doses of a series of strains of streptococcus by a monovalent serum is subject to so many interpretations that the evidence gained cannot be considered of much value in judging accurately the antigenic relationship of the different strains.

The technique observed in the protocols given below has been as follows: The potency of all sera has been titrated for the homologous strain of organism and only the sera which gave a wide range of protection have been used. For infection, virulent streptococci have been used which have been grown for approximately 18 hours in either

plain broth or ascites broth. In the inoculation of animals the technique advised by Neufeld (5) has been followed with only a minor variation. The test animals have been injected intraperitoneally with 0.5 cc. of serum 24 hours before the conduction of the experiment. Tentative trials have shown that if the serum is given simultaneously with the infecting dose, no protection results, and that to insure success the serum must be given at least 8 hours before infection. On the following day a series of virulence controls is inoculated intraperitoneally, and the serum animals are injected in the same manner with doses of cultures ranging from 0.001 to 0.00000001 cc. of broth culture. Animals surviving for a period of 5 days are considered to be adequately protected. By the use of this method, it has been possible to test the antigenic relationship of a considerable number of virulent strains of *Streptococcus hæmolyticus*, and the results of these tests are set forth in the following protocols.

Protocol 1.

In this protocol is shown the titration of the serum of a sheep immunized against Strain S 23. The culture employed for infection was an 18 hour broth culture of No. S 23, which had received eighteen passages through white rats and mice. Each mouse had received 0.5 cc. of immune serum 24 hours previous to infection.

Virulence controls.		Protective power of Serum S 23.	
Dose of culture.	Result.	Dose of culture.	Result.
cc.		cc.	
		0.001	S.
		0.0001	D. in 4 days.
0.00001	D.* in 24 hrs.	0.000001	S.
0.000001	" " 24 "	0.0000001	"
0.0000001	" " 24 "	0.00000001	"

* In the tables D. indicates died, S. survived.

Protocol 2.

In this protocol is shown the protective power of Immune Serum S 3 for two virulent strains of the homologous type, for two strains of each of the heterologous types, and for two unclassified strains. The technique was the same as that in the previous protocol.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	0.0000001 cc.
S 3.18*	S 3	S 3 No serum.	D. 14 hrs.	S.	S.	S.	D. 20 hrs. " 60 "
S 149.16 ²	S 3	S 3 No serum.	S.	S.	D. 20 hrs.	D. 23 hrs.	
S 39.3 ²	S 23	S 3 No serum.	D. 16 hrs.	" 16 " " 16 "	" 7 " " 16 "	" 19 " " 21 "	
S 67.7 ²	S 23	S 3 No serum.	D. 8 hrs.	" 8 "	" 17 "	" 17 " " 23 "	D. 19 hrs. " 21 "
S 128.14 ²	S 60	S 3 No serum.	D. 24 hrs.	D. 24 hrs. " 24 "	D. 48 hrs. " 36 "	" 24 " " 36 "	
S 60.10 ²	S 60	S 3 No serum.	D. 18 hrs.	" 36 " " 18 "	" 36 " S.	" 20 " " 24 "	
S 1.8 ¹	S 84	S 3 No serum.	D. 22 hrs.	" 22 " " 21 "	D. 22 hrs. " 21 "	" 22 " " 21 "	
S 84.17 ²	S 84	S 3 No serum.	" 13 "	" 19 "	" 19 "	" 19 " " 18 "	D. 20 hrs. " 60 "
S 24.25 ³	Unclassified.	S 3 No serum.	D. 14 hrs.	D. 16 hrs.	D. 19 hrs.	D. 21 hrs. " 19 " " 28 "	
S 266.6 ²	"	S 3 No serum.	D. 16 hrs.	D. 16 hrs. " 17 "	D. 19 hrs. " 21 "	" 21 " " 24 "	

* The integer indicates serial number of the culture, the decimal shows the number of animal passages, and the exponent the number of transplants since the last animal passage.

Protocol 3.

In this protocol is shown the protective power of antistreptococcus serum, Type S 23, against two strains of the homologous type, against two strains of each of the heterologous types, and against two unclassified strains. The technique was the same as that employed in the previous protocols.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.000001 cc.	0.0000001 cc.	
S 23.18 ²	S 23	S 23 No serum.	D. 11 hrs.	S. D. 19 hrs.	S. D. 24 hrs.	S. D. 20 hrs.	
S 107.12 ²	S 23	S 23 No serum.	S.	" 16 " " 24 "	S. D. 24 hrs.	S. D. 24 hrs.	
S 3.21 ²	S 3	S 23 No serum.	D. 18 hrs.	" 18 " " 18 "	" 36 " " 36 "	" 64 " " 36 "	
S 80.7 ²	S 3	S 23 No serum.	D. 18 hrs.	" 64 " S.	" 96 " " 36 "	S. D. 36 hrs.	
S 55.22 ¹	S 60	S 23 No serum.	D. 20 hrs.	D. 20 hrs. " 22 "	" 30 " " 20 "	" 30 " " 20 "	
S 60.10 ¹	S 60	S 23 No serum.	D. 20 hrs.	" 20 " " 20 "	S. D. 36 hrs.	S. D. 36 hrs.	
S 84.18 ¹	S 84	S 23 No serum.	D. 12 hrs.	" 18 " " 16 "	" 36 " " 20 "	D. 18 hrs. S.	
S 50.4 ²	S 84	S 23 No serum.	D. 18 hrs.	" 18 " " 18 "	" 18 " " 36 "	D. 18 hrs. " 18 "	
S 276.31 ²	Unclassified.	S 23 No serum.	D. 16 hrs.	" 66 " " 18 "	" 40 " " 40 "	" 40 " " 40 "	
S 24.31 ²	"	S 23 No serum.	D. 16 hrs.	" 16 " " 16 "	D. 20 hrs.	D. 20 hrs.	

Protocol 4.

In this protocol is shown the protective power of antistreptococcus serum, Type S 60, against two strains of homologous type, against two strains of each of the heterologous types, and against two unclassified strains. The technique was the same as that employed in the previous protocols.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	0.0000001 cc.
S 60.10 ²	S 60	S 60 No serum.	D. 19 hrs.	S. D. 17 hrs.	S. D. 19 hrs.	S. D. 19 hrs.	
S 55.22 ²	S 60	S 60 No serum.	S.	S. D. 23 hrs.	S. D. 16 hrs.	S. D. 16 hrs.	
S 3.22 ²	S 3	S 60 No serum.	D. 18 hrs.	D. 18 hrs.	" 18 " " 18 "	" 30 " " 18 "	D. 24 hrs. " 18 "
S 80.8 ²	S 3	S 60 No serum.	D. 18 hrs.	D. 18 hrs.	" 18 " " 18 "	" 22 " " 18 "	" 24 " " 18 "
S 75.6 ¹	S 23	S 60 No serum.	D. 18 hrs.	D. 21 hrs. " 23 "	" 20 " " 17 "	D. 23 hrs.	
S 23.19 ²	S 23	S 60 No serum.	D. 18 hrs.	" 18 "	" 18 " " 18 "	" 24 " " 60 "	D. 60 hrs. " 18 "
S 84.12 ¹	S 84	S 60 No serum.	D. 18 hrs.	D. 18 hrs. " 18 "	" 18 " " 18 "	" 22 " " 20 "	
S 50.5 ³	S 84	S 60 No serum.	D. 18 hrs.	" 18 "	" 18 " " 18 "	" 18 " " 18 "	D. 18 hrs. " 18 "
S 24.28 ²	Unclassified.	S 60 No serum.	D. 17 hrs.	D. 17 hrs.	" 17 " " 17 "	" 24 " " 65 "	D. 31 hrs.
S 276.31 ²	"	S 60 No serum.	D. 9 hrs.	D. 20 hrs. " 23 "	" 24 " " 48 "	" 41 " " 33 "	

In this protocol is shown the protective power of antistreptococcus serum, Type S 84, against two strains of the homologous type, against two strains of each of the heterologous types, and against two unclassified strains. The technique was the same as that employed in previous protocols.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	0.0000001 cc.
S 84.16 ¹	S 84 ¹	S 84 No serum.	S. D. 16 hrs.	S. D. 26 hrs.	S. D. 54 hrs.	S. D. 54 hrs.	0.0000001 cc.
S 20.5 ¹	S 84	S 84 No serum.	S. D. 30 hrs.	S. D. 55 hrs.	S. D. 55 hrs.	S. D. 55 hrs.	
S 3.16 ¹	S 3	S 84 No serum.	" 17 " " 17 "	" 20 " " 20 "	" 29 " " 29 "	" 29 " " 29 "	
S 14.24 ¹	S 3	S 84 No serum.	" 16 " D. 16 hrs.	" 16 " D. 16 hrs.	" 16 " D. 16 hrs.	" 16 " D. 16 hrs.	D. 16 hrs. " 40 "
S 23.16 ¹	S 23	S 84 No serum.	D. 18 hrs. S.	D. 33 hrs. " 22 "	" 32 " " 23 "	" 32 " " 23 "	
S 107.7 ¹	S 23	S 84 No serum.	D. 18 hrs.	" 24 " " 18 "	S. D. 18 hrs.	S. D. 18 hrs.	D. 24 hrs.
S 128.14 ²	S 60	S 84 No serum.	D. 24 hrs.	" 24 " " 24 "	" 24 " " 36 "	" 24 " " 36 "	
S 60.10 ²	S 60	S 84 No serum.	D. 18 hrs. " 18 "	" 18 " S.	" 21 " " 24 "	" 21 " " 24 "	
S 152.5 ¹	Unclassified.	S 84 No serum.	D. 20 hrs.	D. 29 hrs. S.	" 76 " " 28 "	" 76 " " 28 "	
S 266.5 ²	"	S 84 No serum.	D. 15 hrs.	D. 23 hrs. " 38 "	" 45 " " 21 "	" 45 " " 21 "	

Consideration of the above protocols shows that the type relationships manifest from the agglutination reactions have been substantiated by the evidence obtained from the protection tests. As a matter of fact, each reaction has been used to supplement the other, the first clue as to the position of an organism sometimes being obtained by protection and sometimes by agglutination. On the whole, we are inclined to place greater confidence in the reaction of protection than in that of agglutination, and would be slow to draw general conclusions concerning type specificity from agglutination alone with such a variable organism as streptococcus, unless the results of this test could be confirmed by some other specific reaction such as protection. The sera prepared, as is seen from the protocols, have afforded a high degree of protection to white mice against infection with organisms of the homologous type. Little or no protection results when serum of one type is employed against organisms of heterologous types. There are, of course, some exceptions to this general rule. Occasionally strains of *Streptococcus hæmolyticus* are encountered against which all type sera afford a varying degree of protection, and sometimes a serum is obtained from one strain which will protect against an organism of another type, and when the reaction is reciprocally reversed no protection results. At present our knowledge is insufficient to discuss these intermediate reactions intelligently, and their elucidation must await further development of the technique. In all it has been possible to raise the virulence of 31 strains to a point where protection experiments could be performed. Of these, 7 belonged to Type S 3, 8 to Type S 23, 6 to Type S 60, 7 to Type S 84, and 3 to the unclassified group. In view of the difficulty of raising the virulence of the organisms it has been found advantageous to perform the reaction in two ways: first, to test a single monovalent serum against a number of strains; and second, to test a number of sera prepared from strains of the same type against a single virulent strain of that type. In Tables XI to XIV is shown a summary of the total number of protection experiments performed.

TABLE XI.

Summary of the Protective Power of Antistreptococcus Serum, Type S 3, against Strains of the Homologous and Heterologous Types.

Type of serum.	Strain of <i>S. hemolyticus</i> used for production of serum.	Strain of <i>S. hemolyticus</i> and type used for infection of mice.	Minimal lethal dose of <i>S. hemolyticus</i> used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 3	S 3 (Rabbit 1).	S 3 (Type S 3).	0.00000001	S. 0.0001 cc.
S 3	S 3 (" 1).	S 14 (" S 3).	0.0000001	" 0.001 "
S 3	S 3 (Dog 1).	S 3 (" S 3).	0.00000001	" 0.001 "
S 3	S 3 (" 1).	S 95 (" S 3).	0.00001	" 0.01 "
S 3	S 3 (" 1).	S 80 (" S 3).	0.000001	" 0.001 "
S 3	S 3 (" 1).	S 149 (" S 3).	0.000001	" 0.01 "
S 3	S 3 (" 1).	S 146 (" S 3).	0.000001	" 0.01 "
S 3	S 3 (" 1).	S 144 (" S 3).	0 00001	" 0.001 "
S 3	S 111 (Rabbit 2).	S 3 (" S 3).	0.0000001	" 0.001 "
S 3	S 118 (" 3).	S 3 (" S 3).	0.0000001	" 0.0001 "
S 3	S 2 (" 4).	S 3 (" S 3).	0.0000001	" 0.001 "
S 3	S 11 (" 5).	S 3 (" S 3).	0.0000001	" 0.001 "
S 3	S 29 (" 6).	S 3 (" S 3).	0.00000001	" 0.0001 "
S 3	S 16 (" 7).	S 3 (" S 3).	0.00000001	" 0.0001 "
S 3	S 3 (" 1).	S 107 (" S 23).	0.0000001	D. 0.0000001 "
S 3	S 3 (" 1).	S 23 (" S 23).	0.0000001	" 0.0000001 "
S 3	S 3 (Dog 1).	S 27 (" S 23).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 67 (" S 23).	0.00000001	" 0.0000001 "
S 3	S 3 (" 1).	S 39 (" S 23).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 75 (" S 23).	0.000001	" 0.00001 "
S 3	S 3 (" 2).	S 56 (" S 23).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 128 (" S 60).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 60 (" S 60).	0.000001	" 0.000001 "
S 3	S 3 (Rabbit 1).	S 84 (" S 84).	0.00000001	" 0.00000001 "
S 3	S 3 (" 1).	S 1 (" S 84).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 24 (unclassified).	0.00000001	" 0.00000001 "
S 3	S 3 (" 1).	S 276 (").	0.0000001	S. 0.0000001 "
S 3	S 3 (" 1).	S 61 (").	0.00001	" 0.00001 "
S 3	S 3 (Dog 1).	S 152 (").	0.000001	D. 0.000001 "
S 3	S 3 (" 1).	S 266 (").	0.000001	" 0.000001 "
Unclassified.	S 24 (Rabbit 8).	S 3 (Type S 3).	0.000001	" 0.000001 "
"	S 24 (Sheep 2).	S 14 (" S 3).	0.00000001	S. 0.00001 "
"	S 276 (Rabbit 9).	S 14 (" S 3).	0.000001	" 0.000001 "

TABLE XII.

Summary of the Protective Power of Antistreptococcus Serum, Type S 23, against Strains of the Homologous and Heterologous Types.

Type of serum.	Strain of <i>S. hemolyticus</i> used for production of serum.	Strain of <i>S. hemolyticus</i> and type used for infection of mice.	Minimal lethal dose of <i>S. hemolyticus</i> used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 23	S 23 (Sheep 1).	S 23 (Type S 23).	0.0000001	S. 0.001 cc.
S 23	S 23 (" 1).	S 107 (" S 23).	0.0000001	" 0.001 "
S 23	S 23 (" 1).	S 27 (" S 23).	0.000001	" 0.0001 "
S 23	S 23 (" 1).	S 75 (" S 23).	0.0000001	" 0.001 "
S 23	S 23 (" 1).	S 65 (" S 23).	0.0000001	" 0.001 "
S 23	S 23 (" 1).	S 3 (" S 3).	0.0000001	D. 0.000001 "
S 23	S 23 (" 1).	S 80 (" S 3).	0.0000001	S. 0.0000001 "
S 23	S 23 (" 1).	S 55 (" S 60).	0.000001	D. 0.000001 "
S 23	S 23 (" 1).	S 60 (" S 60).	0.000001	S. 0.000001 "
S 23	S 23 (" 1).	S 128 (" S 60).	0.000001	D. 0.000001 "
S 23	S 23 (" 1).	S 50 (" S 84).	0.0000001	" 0.0000001 "
S 23	S 23 (" 1).	S 84 (" S 84).	0.000001	" 0.0000001 "
S 23	S 23 (" 1).	S 24 (unclassified).	0.000001	" 0.00001 "
S 23	S 23 (" 1).	S 276 (").	0.000001	" 0.000001 "
Unclassified.	S 24 (" 2).	S 27 (Type S 23).	0.000001	" 0.000001 "
"	S 24 (" 2).	S 56 (" S 23).	0.000001	" 0.000001 "
"	S 24 (" 2).	S 107 (" S 23).	0.000001	" 0.000001 "
"	S 24 (" 2).	S 39 (" S 23).	0.00000001	" 0.000001 "
"	S 24 (" 2).	S 23 (" S 23).	0.00000001	" 0.0000001 "
"	S 276 (Rabbit 9).	S 27 (" S 23).	0.000001	" 0.000001 "
"	S 276 (" 9).	S 56 (" S 23).	0.00001	" 0.000001 "
"	S 276 (" 9).	S 107 (" S 23).	0.0000001	" 0.0000001 "
"	S 276 (" 9).	S 39 (" S 23).	0.000001	" 0.000001 "
"	S 276 (" 9).	S 23 (" S 23).	0.00000001	" 0.00000001 "
"	S 276 (" 9).	S 67 (" S 23).	0.000001	" 0.000001 "

TABLE XIII.

Summary of the Protective Power of Antistreptococcus Serum, Type S 60, against Strains of the Homologous and Heterologous Types.

Type of serum.	Strain of <i>S. hæmolyticus</i> used for production of serum.	Strain of <i>S. hæmolyticus</i> and type used for infection of mice.	Minimal lethal dose of <i>S. hæmolyticus</i> used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 60	S 128 (Rabbit 10).	S 128 (Type S 60).	0.00001	S. 0.01 cc.
S 60	S 128 (" 10).	S 60 (" S 60).	0.000001	" 0.0001 "
S 60	S 128 (" 10).	S 55 (" S 60).	0.000001	" 0.001 "
S 60	S 128 (" 10).	S 4 (" S 60).	0.000001	" 0.001 "
S 60	S 128 (" 10).	S 72 (" S 60).	0.00001	" 0.001 "
S 60	S 128 (" 10).	S 267 (" S 60).	0.000001	" 0.001 "
S 60	S 128 (" 10).	S 3 (" S 3).	0.0000001	D. 0.0000001 "
S 60	S 128 (" 10).	S 80 (" S 3).	0.0000001	" 0.0000001 "
S 60	S 128 (" 10).	S 75 (" S 23).	0.000001	" 0.00001 "
S 60	S 128 (" 10).	S 65 (" S 23).	0.000001	S. 0.00001 "
S 60	S 128 (" 10).	S 23 (" S 23).	0.0000001	D. 0.0000001 "
S 60	S 128 (" 10).	S 84 (" S 84).	0.000001	" 0.000001 "
S 60	S 128 (" 10).	S 50 (" S 84).	0.0000001	" 0.0000001 "
S 60	S 128 (" 10).	S 24 (unclassified).	0.0000001	" 0.000001 "
S 60	S 128 (" 10).	S 276 (").	0.000001	" 0.000001 "
Unclassified.	S 24 (" 8).	S 128 (Type S 60).	0.000001	S. 0.001 "
"	S 276 (" 9).	S 128 (" S 60).	0.000001	" 0.0001 "

TABLE XIV.

Summary of the Protective Power of Antistreptococcus Serum, Type S 84, against Strains of the Homologous and Heterologous Types.

Type of serum.	Strain of <i>S. hemolyticus</i> used for production of serum.	Strain of <i>S. hemolyticus</i> and type used for infection of mice.	Minimal lethal dose of <i>S. hemolyticus</i> used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 84	S 84 (Sheep 3).	S 84 (Type S 84).	0.000001	S. 0.001 cc.
S 84	S 84 (" 3).	S 1 (" S 84).	0.000001	" 0.0005 "
S 84	S 84 (" 3).	S 20 (" S 84).	0.000001	" 0.001 "
S 84	S 84 (" 3).	S 50 (" S 84).	0.000001	" 0.0005 "
S 84	S 84 (" 3).	S 139 (" S 84).	0.000001	" 0.001 "
S 84	S 84 (" 3).	S 110 (" S 84).	0.0000001	" 0.001 "
S 84	S 84 (" 3).	S 15 (" S 84).	0.0000001	" 0.001 "
S 84	S 1 (Rabbit 11).	S 1 (" S 84).	0.00000001	" 0.00001 "
S 84	S 1 (" 11).	S 84 (" S 84).	0.00000001	" 0.0001 "
S 84	S 1 (" 11).	S 20 (" S 84).	0.000001	" 0.001 "
S 84	S 84 (Sheep 3).	S 3 (" S 3).	0.000001	D. 0.000001 "
S 84	S 84 (" 3).	S 14 (" S 3).	0.00000001	S. 0.000001 "
S 84	S 84 (" 3).	S 23 (" S 23).	0.0000001	D. 0.0000001 "
S 84	S 84 (" 3).	S 107 (" S 23).	0.0000001	S. 0.0000001 "
S 84	S 84 (" 3).	S 39 (" S 23).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 67 (" S 23).	0.000001	D. 0.000001 "
S 84	S 84 (" 3).	S 27 (" S 23).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 56 (" S 23).	0.000001	" 0.000001 "
S 84	S 1 (Rabbit 11).	S 107 (" S 23).	0.0000001	S. 0.00000001 "
S 84	S 1 (" 11).	S 128 (" S 60).	0.000001	D. 0.000001 "
S 84	S 84 (Sheep 3).	S 128 (" S 60).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 60 (" S 60).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 276 (unclassified).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 277 (").	0.000001	S. 0.000001 "
S 84	S 84 (" 3).	S 152 (").	0.000001	D. 0.000001 "
S 84	S 84 (" 3).	S 266 (").	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 24 (").	0.000001	" 0.000001 "
Unclassified.	S 24 (Rabbit 8).	S 84 (Type S 84).	0.000001	" 0.000001 "
"	S 24 (Sheep 2).	S 1 (" S 84).	0.000001	" 0.000001 "
"	S 276 (Rabbit 9).	S 84 (" S 84).	0.00000001	" 0.00000001 "
"	S 276 (" 9).	S 20 (" S 84).	0.000001	" 0.000001 "
"	S 276 (" 9).	S 50 (" S 84).	0.000001	" 0.000001 "

DISCUSSION.

The complete biological classification of any pathogenic micro-organism presents a very complex problem. The first phase of the undertaking concerns itself with the development of reliable methods for the determination of antigenic differences between members of the species and the application of these methods to the discovery of the immunological relationships between a limited number of strains purposefully selected. In this way the degree of similarity and diversity of type is shown and also the probable number of types, and the proportion of classifiable to unclassifiable strains. The next step of necessity is the testing of the adequacy and universality of the information so gained by applying the tentative classification to a large number of strains of the organism obtained under what may be described as normal conditions of pathogenicity. That some sort of equilibrium has been established in nature among microorganisms that have produced disease over long periods of time is not unlikely. Indeed, evidence obtained from the study of pneumococci supports this view (7), although departure from the norm may occur under special conditions (8). After the relationships of the pathogens of the species to one another have been discovered, it then becomes important for purposes of epidemiological study to compare by the same methods the pathogenic with the saprophytic varieties. This task requires years for its completion and many difficulties and seemingly unexplainable phenomena are encountered. In the beginning, the broader lines of differentiation must be drawn, and divergent results discarded for the time being, since, if the original conception is correct, most of the discrepancies disappear with the advance of knowledge.

In this paper are presented the facts so far obtained in the present study of *Streptococcus hemolyticus* in accordance with the plan outlined above. The strains were collected in a limited community during the course of what may be considered an epidemic of bronchopneumonia secondary to measles. Individuals, however, from all parts of the United States were passing rapidly through this community which was a center for primary training of the aviation service, so that a wider range of territory is represented than the im-

mediate community itself. All the strains were investigated as to their cultural reactions, bile solubility, capacity to hemolyze red blood cells and to ferment the different test sugars, and as to hydrogen ion concentration limiting their growth, and thus identified as accurately as possible as *Streptococcus hæmolyticus* of the human type.

A technique was then developed for studying the immunological reactions of agglutination and protection. By the reaction of agglutination four distinct immunological types and a certain number of unclassifiable strains have been discovered among the 125 strains studied. Individuals of the same type are closely related to one another immunologically, and the different types can be sharply distinguished one from the other. In addition to the four types, study of the reactions of which has been completed, there are in addition two other types, investigation of which is as yet incomplete. The technique of the agglutination reaction demands great care, both as regards the handling of the organisms and the preparation of the medium for their growth. In the medium used by us, a large percentage of strains has grown sufficiently diffusely to permit the preparation of stable suspensions. To what extent continuous growth in this medium has promoted the tendency to diffuseness, and whether the same percentage of freshly isolated strains will grow diffusely, we are as yet unable to say. We have found that by the immunization of sheep a highly specific agglutinating serum is obtained, but that the serum produced from rabbits is not so specific and may show a wider range of crossing, especially in one of the types of streptococcus described. Variations in the specificity of different animal sera have been observed by students of the immunological reactions of meningococcus. In order fully to understand this phenomenon, it would be necessary to compare the specificity of immune sera produced from different species of animals by means of the method of absorption. It is not as yet possible to undertake this kind of an investigation of *Streptococcus hæmolyticus*. The observation has been made, however, that rabbit sera showing non-specific cross-agglutination reactions in general fail to manifest corresponding cross-protection reactions.

Whenever it has been possible to raise the animal virulence of strains of *Streptococcus hæmolyticus*, the evidence obtained from the agglutination tests has been confirmed by that gained from the pro-

tection reaction. In all instances in which this has been done, one reaction has corroborated the findings of the other. The performance of reliable protection tests has been made possible by the production of sufficiently high titer antistreptococcus sera, and by the possibility of raising the animal virulence of a certain number of strains to a high degree. The types of *Streptococcus hæmolyticus* have been noted as Types S 3, S 23, S 60, and S 84, from the serial numbers of the representative strains. This nomenclature is not put forth as a final one, since we realize that probably many other human types exist, to say nothing of the bovine and cheese varieties, and that the proportional distribution of the different varieties pathogenic for man may be very different from that represented by this work. Streptococcus is the largest of all pathogenic groups of bacteria and many years will be required to bring out the information necessary to the perfecting of an adequate classification.

It is of considerable interest that all the members of Type S 60 ferment mannite, and that none of the members of the other groups so far encountered ferments this sugar. A few unclassifiable strains, however, have been found to be mannite fermenters.

This work has cleared up a number of points about *Streptococcus hæmolyticus* which have been in dispute for many years. In the first place, *Streptococcus hæmolyticus* of human origin is not a unit type as was previously supposed, but probably consists of a number of types, at least four of which have been definitely identified. Previous investigators have stated that freshly isolated human strains change their antigenic properties on animal passage, and that the latter procedure for the development of animal virulence gives a common antigenic character to all strains. We have found no evidence to support this contention; in fact immune sera produced with human strains that have never been passed through animals afford a high degree of protection against strains that have received many animal passages. In addition, the antigenic differences between strains of *Streptococcus hæmolyticus* which have been passed through animals are as distinct as those between strains which have not been so passed. The types of *Streptococcus hæmolyticus* studied have been obtained almost exclusively from the respiratory tract and from a limited source of supply, and there is some reason to believe that those which pro-

duce cellulitis, erysipelas, and septicemia may be of somewhat different character. It is, therefore, readily seen that only a beginning has been made in the classification of *Streptococcus hæmolyticus*, and that before the classification is complete and the relative dominance of the different pathogenic varieties determined, much work must be done.

SUMMARY.

1. Immunological differences have been shown to exist between strains of *Streptococcus hæmolyticus* of the human type.

2. Four biological types have been identified by means of the reactions of agglutination and protection.

3. At least two other types have been encountered and the indications are that more exist.

BIBLIOGRAPHY.

1. Schottmüller, H., *Münch. med. Woch.*, 1903, I, 849.
2. Andrews, F. W., and Horder, T. J., *Lancet*, 1906, ii, 708. Holman, W. L., *J. Med. Research*, 1916, xxxiv, 377. Blake, F. G., *ibid.*, 1917, xxxvi, 99.
3. Smith, T., and Brown, J. H., *J. Med. Research*, 1914-15, xxxi, 455. Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 253. Ayers, S. H., *J. Bacteriol.*, 1916, i, 84. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.
4. Smillie, W. G., *J. Infect. Dis.*, 1917, xx, 45. Cole, R., and MacCallum, W. G., *J. Am. Med. Assn.*, 1918, lxx, 1146. Levy, R. L., and Alexander, H. L., *ibid.*, 1918, lxx, 1827. Opie, E. L., Freeman, A. W., Blake, F. G., Small, J. C., and Rivers, T. M., *ibid.*, 1919, lxxii, 556.
5. Marmorek, A., *Ann. Inst. Pasteur*, 1895, ix, 593. Aronson, H., *Deutsch. med. Woch.*, 1903, xxix, 439. Neufeld, F., *Z. Hyg. u. Infektionskrankh.*, 1903, xlv, 161. Kinsella, R. A., and Swift, H. F., *J. Exp. Med.*, 1918, xxviii, 169. Howell, K., *J. Infect. Dis.*, 1918, xxii, 230.
6. Gay, F. P., *J. Lab. and Clin. Med.*, 1918, iii, 721.
7. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1915, xxi, 114.
8. Lister, F. S., *Publications South African Inst. Med. Research*, No. 2, 1913; No. 8, 1916; No. 10, 1917.

THE OXYGEN OF THE ARTERIAL AND VENOUS BLOOD IN PNEUMONIA AND ITS RELATION TO CYANOSIS.

By WILLIAM C. STADIE, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

PLATES 13 TO 15.

(Received for publication, May 14, 1919.)

In the recent epidemic of influenza with its accompanying pneumonia the unusual frequency of cyanosis was striking, and was in fact one outstanding feature of the epidemic. To find the cause, if possible, of the cyanosis a group of pneumonia cases at the Hospital of The Rockefeller Institute was studied during the past winter, with particular reference to cyanosis and its relation to the arterial and venous blood oxygen.

Studies of the venous blood have been made, notably by Lundsgaard¹ in cardiac insufficiency, and Harrop² in pneumonia; but the interpretation of the results is difficult, because of the undeterminable factors which affect the venous oxygen, such as variations in the rates of circulation and metabolism in the parts from which the blood is drawn. It appeared that satisfactorily complete data on which to base an explanation of the cyanosis could be expected only from analyses of arterial as well as venous blood. A group of 33 cases of pneumonia is here presented in which the oxygen of both arterial and venous blood has been determined.

Method.

Technique of Arterial Puncture.—Hürter³ has shown that puncture of the radial artery is a safe procedure. At the Hospital of

¹ Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxiii, 133; *J. Exp. Med.*, 1918, xxvii, 179, 219.

² Harrop, G. A., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 10.

³ Hürter, *Deutsch. Arch. klin. Med.*, 1912, cviii, 1.

The Rockefeller Institute arterial puncture has been done six times on one patient without injury. In all, about 90 punctures have been done and no ill results have been observed. The possible dangers are hemorrhage, thrombosis, embolism, or aneurysm, but in this series these have never been observed, even after 4 to 5 months observation. Occasionally if proper precautions are not observed, an undue amount of blood extravasation occurs; this has been observed here once, but the blood was rapidly absorbed and no after effects were seen.

An ordinary 20 cc. all glass Luer syringe is used with a Luer needle 1 to 2 mm. in diameter. The point of the needle is beveled at an angle of about 45° and must be very sharp. To prevent the blood from coming in contact with air, 1 or 2 cc. of sterile albolene are poured into the barrel of the syringe, the plunger is inserted, and the syringe with the attached needle inverted. The plunger is forced upward, and the air in the dead space at the distal end of the syringe and needle is expelled. The excess of albolene is then forced out so that only a small amount remains in the needle and in the small dead space. The patient's arm is laid horizontally upon a pillow, the hand is flexed backwards, and the region over the radial artery is sterilized with tincture of iodine. The end of the left index finger of the operator is then sterilized with iodine and, by using this finger in palpation, the best site for the puncture is determined. (Since the position of the artery is determined solely by palpation, it is advantageous to avoid gloves and use the bare finger.) The skin at the site of the proposed puncture is anesthetized with novocaine. The syringe and needle are held at an angle of about 45° to the surface, the needle is then pushed through the skin, and, after carefully relocating its position, the needle is entered into the artery. It is essential that the position of the artery should be sharply located and the point of maximum pulsation chosen (usually opposite the radial styloid); then the artery is easily entered, the pressure of the blood stream forces up the plunger so that suction is unnecessary, and within 15 to 60 seconds from 10 to 20 cc. of blood can be obtained. The needle is then quickly withdrawn, and by means of a compress, firm pressure is immediately applied over the artery for 1 or 2 minutes, so as to obliterate it temporarily and prevent extravasation. The

wrist is then bandaged with three or four thicknesses of compress to get greater pressure, and at the end of about 2 to 3 hours the bandage may be removed. If the artery is missed at the first puncture, and especially if a hematoma begins to form or blood extravasates around the needle, the operator should desist at once.

The blood thus collected is transferred to a tube 2.5 by 10 cm. in which a layer of albolene at least 2 cm. deep has previously been placed (to prevent contact with air) with some potassium oxalate to prevent coagulation.

Not all cases are suitable for arterial punctures, especially where repeated punctures are contemplated. Many women and some men have small radial arteries deeply situated, and hence difficult to puncture. The more rapid and bounding the pulse, the easier the puncture; and cases with pulse rates of 70 or below are difficult. Occasionally when the artery has been touched with the point of the needle it becomes almost pulseless, due no doubt to reflex vasoconstriction, but after 15 to 60 seconds it relaxes and the puncture may be finished.

Following the puncture there is a numbness of the radial side of the hand, but this wears off rapidly. About 50 per cent of the subjects complain of very slight dull pain at the wrist lasting about 12 to 24 hours, but this is without significance.

In two cases which were autopsied the radial arteries, which had been punctured several times, were dissected out. In one case the sites of the punctures were difficult to determine, and there was only a small amount of extravasation of blood. In the second case one puncture had been made 4 or 5 hours before death, when the patient was almost pulseless and the puncture difficult; here there was a moderate amount of extravasated blood in the tissues surrounding the artery, and the site of the puncture was marked by a pin-head point of extravasation of blood in the wall of the artery.

Technique of Venous Puncture.—The venous blood was obtained without stasis by the technique devised by Lundsgaard.¹

*Determination of Oxygen Content, Oxygen Capacity, and Oxygen Unsaturation.*¹—The method of Van Slyke⁴ was used. The arterial

⁴ Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.

TABLE I.
Determination of Oxygen Content, Oxygen Capacity, and Oxygen Unsaturation.

Case No.	Day of disease.	Diagnosis and clinical notes.	Result.	Cyanosis.					Oxygen content.		Oxygen capacity per 100 cc. of blood.	Oxygen unsaturation.			
				General.	Cheeks.	Nose.	Lips.	Ears.	Fingers.	Arterial, per 100 cc. of blood.	Venous, per 100 cc. of blood.	Arterial.		Venous.	
												Per 100 cc. of blood.	Per cent.	Per 100 cc. of blood.	Per cent.
3	6th	Lobar pneumonia.	R.*	0†	0	0	0	0	0	cc.	cc.	cc.		cc.	
	8th	Acutely ill.		0	0	0	0	0	0	18.4	14.8	17	8.5	5.3	26.4
	11th			2	1	0	0	0	2	17.7	15.0	20.1†	2.4	5.1	25.4
	14th	After crisis; much better.		1	1	0	0	0	1	19.1	17.5	20.6	2.9	14.1	
	20th	Convalescing.		0	0	0	0	0	0	18.9	15.1	21.0	1.9	3.5	16.7
	30th	"		0	0	0	0	0	0	19.9	17.8	20.1	1.2	5.0	24.9
4	7th	Influenza; bronchopneumonia. Extremely ill.	D.	3	3	2	1	1	3	15.1	5.3	20.3	5.2	15.0	73.9
5	10th	Influenza; bronchopneumonia. Heart's blood, 5 p.m.	"	4	4	4	4	4	4		3.6	23.2		19.6	84.5
8	24th	Influenza; bronchopneumonia.	R.	0	0	0	0	0	0	17.1	12.0	18.6	1.5	6.6	35.5
9	9th	Bronchopneumonia.	D.	4	4	3	1	2	4	7.9	3.6	24.8	16.9	21.2	85.5
10	18th	"	R.	0	0	0	0	0	0	22.7	18.5	23.8	1.1	4.6	22.3
11	7th	Influenza; bronchopneumonia; pleuritic effusion.	"	2	2	0	0	1	2	23.1	20.1	25.7	2.6	10.1	21.8
	22nd			0	0	0	0	0	0	21.5	16.1	23.6	2.1	8.9	31.8

14	9th	Lobar pneumonia; influenza.	D.	3	2	1	1	1	3	21.1	10.6	26.6	5.5	20.7	16.0	60.2
15	3rd 11th	Influenza; bronchopneumonia	R.	1	1	0	0	0	1	20.0	16.7	21.7	1.7	7.8	5.0	23.0
				0	0	0	0	0	0	19.1	16.4	20.6	1.5	7.3	4.2	20.4
16	4th	Bronchopneumonia.	D.	3	3	2	0	2	3	20.7	13.8	24.1	3.4	14.1	10.3	42.8
17	13th 15th	Lobar pneumonia.	R.	1	1	0	0	0	1	16.9	16.2	20.2	3.3	16.3	4.0	19.8
				1	1	1	1	0	0	16.1	9.0	18.2	2.1	11.5	10.2	56.0
18	23rd 24th 26th	" Influenza; empyema.	D.	2	2	1	1	0	2	19.6	17.7	23.5	3.9	16.6	5.8	24.7
				3	3	2	1	2	3	19.8	17.1	23.7	3.9	16.5	6.6	27.8
				4	4	3	2	3	4	14.4	1.3§	23.3	8.9	38.2	22.0	94.5§
19	4th 5th 13th	" bronchopneumonia.	R.	0	0	0	0	0	0	21.0	17.5	22.8	1.8	7.9	5.3	23.2
				0	0	0	0	0	0	21.7	17.2	23.4	1.7	7.3	6.2	26.5
				0	0	0	0	0	0	20.7	17.2	20.7	0.0	0.0	3.5	16.9
20	11th	"	"	1	1	0	1	0	1	18.1	15.7	20.9	2.8	13.4	5.2	24.9
21	7th 7th	" Heart puncture, (p.m.; 1½ hrs.).	D.	4	4	4	2	3	4	10.8	4.7	22.5	11.7	52.0	17.8	79.2
											1.8	22.5			20.7	
22	11th 13th	Lobar pneumonia.	"	2	2	1	1	0	2	18.6	9.3	23.1	4.5	19.5	13.8	59.8
				2	2	1	1	1	2	16.7	12.7	22.3	5.6	25.1	9.6	43.0

* R. indicates recovered, D. died.

† The numbers in this column indicate plus signs; i. e., 0 indicates no cyanosis, 1 indicates + (slight cyanosis), 2 indicates ++ (moderate cyanosis), 3 indicates +++ (marked cyanosis), and 4 indicates ++++ (intense cyanosis).

‡ Determined colorimetrically. (Palmer method—Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119).

§ Postmortem heart's blood.

TABLE I—*Concluded.*

Case No.	Day of disease.	Diagnosis and clinical notes.	Result.	Cyanosis.						Oxygen content.		Oxygen capacity per 100 cc. of blood.	Oxygen unsaturation.			
				General.	Cheeks.	Nose.	Lips.	Ears.	Fingers.	Arterial, per 100 cc. of blood.	Venous, per 100 cc. of blood.		Arterial.		Venous.	
													Per 100 cc. of blood.	Per cent.	Per 100 cc. of blood.	Per cent.
23	6th 7th	Influenza; bronchopneumonia.	R.	1	1	1	1	0	1	18.5	11.3	21.8	3.3	15.1	10.5	48.2
				1	1	1	1	0	1	21.6	9.2	23.7	2.1	8.9	14.5	61.2
24	6th 11th 25th	"	"	0	0	0	0	0	0	17.9	16.7	18.6	0.7	3.8	1.9	10.2
				0	0	0	0	0	0	20.5	11.4	21.0	0.5	2.4	9.6	45.7
				0	0	0	0	0	0	19.1	15.9	19.9	0.8	4.0	4.0	20.1
25	9th	"	D.	3	3	2	1	2	3	15.7	12.6	28.1	12.4	44.1	15.5	55.2
29	14th	Lobar pneumonia.	R.	0	0	0	0	0	0	19.9	14.8	20.4	0.5	2.5	5.6	27.5
30	6th	Influenza; bronchopneumonia.	D.	3	3	1	2	2	3	17.2	7.9	22.9	5.7	24.9	12.0	48.3
31	4th 9th	Multiple pulmonary abscesses; empy-ema.	"	2	1	1	0	1	2	17.1	15.7	20.2	3.1	15.3	4.5	22.3
				1	0	0	0	0	2	17.8		19.6	1.8	9.2		
32	6th 7th	Influenza; bronchopneumonia.	"	1	0	1	1	1	2	20.7	9.7	24.7	4.0	16.2	15.0	60.8
				3	2	2	1	2	4	19.9	11.8	26.2	6.3	24.0	14.4	55.0
33	8th	"	"	2	1	1	0	1	2	17.0	13.5	22.2	5.2	23.4	8.7	39.2
34	10th 13th	Bronchopneumonia.	"	3	3	1	1	2	3	12.9		17.1	4.2	24.6		
				3	3	2	1	2	3	11.3		16.2	4.8	29.6		

35	16th 17th 23rd 38th	Bronchopneumonia.	R.	3 2 3 1	3 3 3 0	1 1 2 0	2 1 2 0	2 3 0 1	3 2 1 1	12.8 13.7 11.7 13.3	10.2 9.7	19.1 17.9 15.8 14.9	6.3 4.2 4.1 1.6	33.0 23.5 25.9 10.7	8.9 8.2	46.7 45.7
36	9th	"	"	1	0	0	0	0	1	22.8	17.7	26.3	3.5	13.3	5.6	21.3
37	7th 8th	"	"	0 0	0 0	0 0	1 0	1 0	1 0	20.5 20.4		21.8 22.0	1.3 1.6	6.0 7.3	3.2	14.4
38	4th	Lobar pneumonia.	"	0	0	0	0	0	1	17.4	12.5	19.0	1.6	8.4	6.5	34.2
39	5th	Bronchopneumonia.	"	2	2	2	2	2	2	18.3	12.9	21.4	3.1	14.5	8.5	39.7
40	7th	"	D.	3	2	2	2	1	3	16.2		22.3	6.1	27.3		
41	9th	"	"	3	2	1	1	3	3	20.7		28.6	7.9	27.6		
42	9th	"	"	4	2	2	3	2	4	8.1		17.8	9.7	54.5		

and venous samples were taken under albolene to prevent contact with air and analyzed in duplicate immediately for oxygen content. A portion of the blood was saturated with oxygen and the total oxygen capacity determined, as described by Van Slyke. Thus are obtained (1) arterial oxygen content (cubic centimeters of oxygen combined with hemoglobin in 100 cc. of arterial blood), (2) venous oxygen content (a similar value for venous blood), (3) total oxygen capacity (cubic centimeters of oxygen combined with the hemoglobin of 100 cc. of blood when fully saturated).

The difference between oxygen content and total oxygen capacity has been named by Lundsgaard¹ the oxygen unsaturation, and we have followed his usage of the term. The unsaturation may be expressed either as cubic centimeters of oxygen per 100 cc. of blood, or as percentage of the total oxygen capacity. In the latter case the data represent the per cent of total hemoglobin in the form of reduced hemoglobin. An example will make this clear.

	cc.	per cent
Arterial oxygen content.....	18.0	90.0
Venous oxygen content.....	14.0	70.0
Total oxygen capacity.....	20.0	100.0
Arterial oxygen unsaturation.....	2.0	10.0
Venous oxygen unsaturation.....	6.0	30.0

RESULTS.

Table I gives the results obtained in the 33 cases of pneumonia studied.

38 cases were studied which were divided as indicated in Table II.

TABLE II.
Classification of Cases.

Diagnosis.	No
Pneumonia, lobar.....	7
Postinfluenzal bronchopneumonia	25
Multiple pulmonary abscesses.....	1
Normal individuals	5
Total.....	38
Complications, empyema.....	3

Results in Normal Controls.—Table III gives the results obtained in five normal resting men. All these subjects were up and about, but were punctured 15 to 30 minutes after resting in bed.

The range of arterial oxygen content is from 17.9 to 22.1 cc. per 100 cc. of blood. The arterial unsaturation varies from 2.8 to 6.3 per cent. The venous unsaturation varies from 22.7 to 33 per cent. The arterial blood is usually assumed to be approximately saturated, but in the five individuals given in Table III the mean value is 95 per cent. The values found for the venous oxygen unsaturation are in close accord with those of Lundsgaard in normal individuals.

TABLE III.

Arterial and Venous Oxygen, Total Oxygen Capacity, and Arterial and Venous Oxygen Unsaturation in Five Normal Individuals.

Individual No.	Oxygen content.		Oxygen capacity per 100 cc. of blood.	Unsaturation.			
	Arterial, per 100 cc. of blood.	Venous, per 100 cc. of blood.		Arterial.		Venous.	
				Per 100 cc. of blood.	Per cent.	Per 100 cc. of blood.	Per cent.
	cc.	cc.	cc.	cc.		cc.	
1	17.9	12.8	19.1	1.2	6.3	6.3	33.0
2	21.0	16.7	21.6	0.6	2.8	4.9	22.7
3	22.1	17.2	23.3	1.2	5.2	6.1	26.2
4	20.2	15.6	21.6	1.4	6.5	6.0	27.8
5	19.5	15.4	20.3	0.8	3.9	4.9	24.1
Mean.....	20.2	15.6	21.2	1.0	5.0	5.6	26.8

Arterial and Venous Oxygen in the Pneumonia Cases.—That the arterial and venous oxygen content and unsaturation in the pneumonia cases show striking contrasts to the normal individuals is seen at once from Table IV.

TABLE IV.

Maximum and Minimum Arterial and Venous Oxygen Content and Arterial and Venous Oxygen Unsaturation in Pneumonia Cases.

Arterial content per 100 cc. of blood.		Arterial unsaturation.		Venous content per 100 cc. of blood.		Venous unsaturation.	
Maximum.	Minimum.	Maximum.	Minimum.	Maximum.	Minimum.	Maximum.	Minimum.
cc.	cc.	per cent	per cent	cc.	cc.	per cent	per cent
22.9	7.9	68.2	0.0	20.1	3.6	85.5	14.4

The arterial oxygen unsaturation ranges up to 68.2 per cent as contrasted to 6.3 per cent for normal individuals. For the venous unsaturation the variations are just as marked.

Oxygen Unsaturation in Fatal and Non-Fatal Cases.—If the cases are divided according to the outcome, equally characteristic differences are obtained. Table V shows that the maximum arterial unsaturation

TABLE V.
Arterial and Venous Oxygen Unsaturation in Sixteen Non-Fatal Cases.

Case No.	Maximum* unsaturation.	
	Arterial.	Venous.
	<i>per cent</i>	<i>per cent</i>
3	14.1	60.1
8	8.1	35.5
10	4.6	22.3
11	10.1	31.8
15	7.8	23.0
17	16.3	56.0
19	9.8	26.5
20	13.4	24.9
23	15.1	61.2
24	4.0	45.7
29	2.5	27.5
35	33.0	46.7
36	13.3	21.3
37	7.5	14.4
38	8.4	34.2
39	14.5	39.7
Mean	13.9	36.3

* Where more than one determination was made the maximum observed value is given.

in the recovered cases is 33 per cent, while the mean value (13.9 per cent) is more than twice the normal mean. In one case (No. 35), however, the arterial unsaturation was 33 per cent. This patient was desperately ill and the outcome for some time appeared hopeless, but she made a remarkable recovery.

Table VI shows that the arterial unsaturation in the fatal cases is much greater, the mean value being 32 per cent. Fourteen of the

sixteen fatal cases, but only one of sixteen non-fatal cases, had an arterial unsaturation greater than 20 per cent. Hence the fatal outcome of pneumonia is usually associated with a great degree of arterial unsaturation, and the arterial oxygen unsaturation offers a valuable prognostic sign. Rarely does a patient with a value greater than 20 per cent recover (one case out of 33).

A study of the venous unsaturation shows a far less degree of uniformity, and indicates the presence of factors difficult to control,

TABLE VI.
Arterial and Venous Oxygen Unsaturation in Sixteen Fatal Cases.

Case No.	Maximum* unsaturation.	
	Arterial.	Venous.
	<i>per cent</i>	<i>per cent</i>
4	25.6	73.9
9	68.2	85.5
14	20.7	60.2
16	14.1	42.8
18	38.2	
21	52.0	79.2
22	25.1	59.8
25	44.1	55.2
30	24.9	48.3
31	15.3	22.3
32	24.0	60.8
33	23.4	39.2
34	29.6	
40	27.3	
41	27.6	
42	54.5	
Mean.....	32.0	57.0

* Where more than one determination was made the maximum observed value is given.

which make the determination of the venous unsaturation of less prognostic significance than the arterial. However, in the fatal cases the mean value is 20 per cent higher than in the non-fatal cases. Eight of the eleven cases, or 72 per cent, showing a venous unsaturation over 47 per cent were fatal, while thirteen out of sixteen, or 81 per cent, showing less than 47 per cent venous unsaturation recovered.

Table VII gives briefly a summary of the above discussion with the maximum and minimum arterial and venous oxygen unsaturation in the fatal and non-fatal cases.

TABLE VII.

Maximum and Minimum Arterial and Venous Oxygen Unsaturation in Fatal and Non-Fatal Cases.

Type of case.	No. of cases.	Arterial unsaturation.			Venous unsaturation.		
		Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Fatal cases.....	16	68.2	14.1	32.0	85.5	22.3	57.0
Non-fatal cases.....	16	33.0	1.6	13.9	61.2	14.4	36.3
Normal individuals..	5	6.5	2.8	5.0	33.0	22.7	26.8

Cyanosis.

Cyanosis may be caused by one of three factors or any combination of these three.

1. *Disturbance of the Capillary Bed.*—If there is a constriction of the arterial precapillaries, or a dilatation of the capillaries due to any cause, there is a stagnation of blood in the capillaries which, in the superficial or distal parts of the body, such as the lips, nose, ears, or fingers, gives cyanosis. For example, the cyanosis after prolonged exposure to cold is undoubtedly due to this cause. Cyanosis associated with various vasomotor paralyses, as in hemiplegia and poliomyelitis, may also be explained in this way.

2. *Change in the Hemoglobin.*—Certain intoxications change the hemoglobin into substances which do not contain labile oxygen, and therefore reduce the oxygen capacity of the blood. These substances are methemoglobin and sulfhemoglobin, and they are found in the blood associated with the cyanosis of acetanilide, phenacetin, potassium chlorate, or nitrobenzol poisoning.⁵

Enterogenous cyanosis due to methemoglobin or sulfhemoglobin, and cases of cyanosis in which the presence of these substances can

⁵ Hammarsten, O., Text book of physiological chemistry, New York, 7th edition, 1914, 283.

be shown, and in which there is a reduction of the total oxygen capacity of the blood, would fall into this class.

It is possible that the cyanosis associated with pneumonia, more particularly in the very severe cases with a marked septicemia, may in some measure be caused by this factor. Butterfield and Peabody⁶ have shown that the growth of pneumococci *in vitro* results in the formation of a substance with the optical properties of methemoglobin. Further, Peabody,⁷ in rabbits in which he induced an overwhelming septicemia with pneumococci, found a rapid and marked fall in the total oxygen capacity of the blood, and also observed that the blood was of a brownish color and took up oxygen slowly. However, he was rarely able to demonstrate the presence of methemoglobin in such blood, and it may be pointed out that such overwhelming septicemias as were produced in his rabbits (direct films of the blood showed numerous organisms) are never found in man. Peabody⁸ and Harrop² found, in a few severe cases of pneumonia, a diminution in the total oxygen capacity of the blood, but were unable to demonstrate the presence of methemoglobin in the blood.

3. *Admixture Cyanosis*.—The third factor in the production of cyanosis is a deficient or incomplete oxygenation of the blood as it passes through the pulmonary capillaries, or the passage of but part of the blood through the lungs (as in congenital heart disease), so that there is an abnormally low percentage of oxyhemoglobin in the peripheral circulation. Obviously the study of the arterial blood oxygen would determine this.

Degree of Cyanosis and the Oxygen Unsaturation.—Cyanosis, to a greater or less degree, was observed in 28 cases out of 33. In order to compare cyanosis in different parts from time to time the following scale was used. \pm indicates very slight, + slight, ++ moderate, +++ marked, and ++++ intense cyanosis.

In the five cases (Nos. 8, 10, 19, 24, and 29) of pneumonia in which no cyanosis was observed at any time, the maximum arterial un-

⁶ Butterfield, E. E., and Peabody, F. W., *J. Exp. Med.*, 1913, xvii, 587.

⁷ Peabody, F. W., *J. Exp. Med.*, 1913, xviii, 1.

⁸ Peabody, F. W., *J. Exp. Med.*, 1913, xviii, 7.

saturation was 8.1 per cent, slightly greater than normal (Table VIII). The mean value is 5.4 per cent, only 0.4 per cent greater than for the normal individuals. The venous unsaturations follow in the same order, but are more irregular.

In the twenty-seven cases showing cyanosis during their illness (Table IX), the difference is at once apparent. Here the variations are from 7.3 to 68.2 per cent of arterial unsaturation, and all but three of the values are above 10 per cent. The mean, 24.7 per cent, is five times greater than in normal individuals. Similarly the values

TABLE VIII.

Maximum Arterial and Venous Oxygen Unsaturation in Cases without Cyanosis.*

Case No.	Arterial unsaturation.		Venous unsaturation.	
	Per 100 cc. of blood.	Per cent.	Per 100 cc. of blood.	Per cent.
	cc.		cc.	
8	1.5	8.1	6.6	35.5
10	1.1	4.6	5.3	22.3
19	1.8	7.9	6.2	26.5
24	0.8	4.0	9.6	45.7
29	0.5	2.5	5.6	27.5
Mean.....	1.1	5.4	6.7	31.5

*Where more than one determination was made the maximum observed value is given.

for the venous unsaturation are higher and the mean, 44.5 per cent, is greater than in the cases without cyanosis.

Not only is there a greatly increased arterial and venous unsaturation in the cases with cyanosis, but there is a definite relation between the degree of cyanosis and the degree of unsaturation. In Table X the observations are divided into five groups according to whether there was no, slight, moderate, marked, or intense cyanosis at the time the blood was obtained. There is a gradual increase in both arterial and venous unsaturation as the associated cyanosis increases. This is strikingly brought out by Text-fig. 1 which shows as a curve the relation between unsaturation and cyanosis. The curve for the venous unsaturation parallels that for the arterial unsaturation.

TABLE IX.

Maximum Arterial and Venous Oxygen Unsaturation in Cases with Cyanosis.*

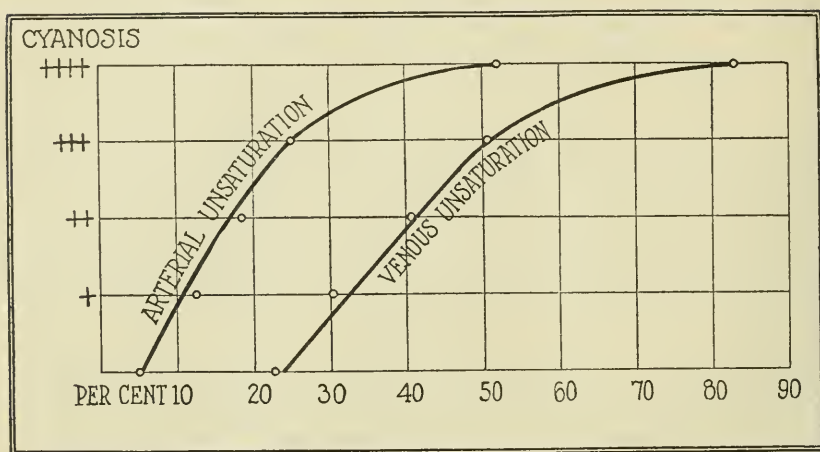
Case No.	Arterial unsaturation.		Venous unsaturation.	
	Per 100 cc. of blood.	Per cent.	Per 100 cc. of blood.	Per cent.
	cc		cc.	
3	2.9	14.1	5.3	26.4
4	5.2	25.6	15.0	73.9
9	16.9	68.2	21.2	85.5
11	2.6	10.1	5.6	21.8
14	5.5	20.7	16.0	60.2
15	1.7	7.8	5.0	23.0
16	3.4	14.1	10.3	42.8
17	3.3	16.3	10.2	56.0
18	8.9	38.2	6.6	27.8
20	2.8	13.4	5.2	24.9
21	11.7	52.0	17.8	79.2
22	5.6	25.1	13.8	59.8
23	3.3	15.1	14.5	61.2
25	12.4	44.1	15.5	55.2
30	5.7	24.9	12.0	48.3
31	3.1	15.3	4.5	22.3
32	6.3	24.0	15.0	60.8
33	5.2	23.4	8.7	39.2
34	4.8	29.6		
35	6.3	33.0	8.9	46.7
36	3.5	13.3	5.6	21.3
37	1.6	7.3	3.2	14.4
38	1.6	8.4	6.5	34.2
39	3.1	14.5	8.5	39.7
40	6.1	27.3		
41	7.9	27.6		
42	9.7	54.5		
Mean.....	5.6	24.7	10.2	44.5

* Where more than one determination was made the maximum observed value is given.

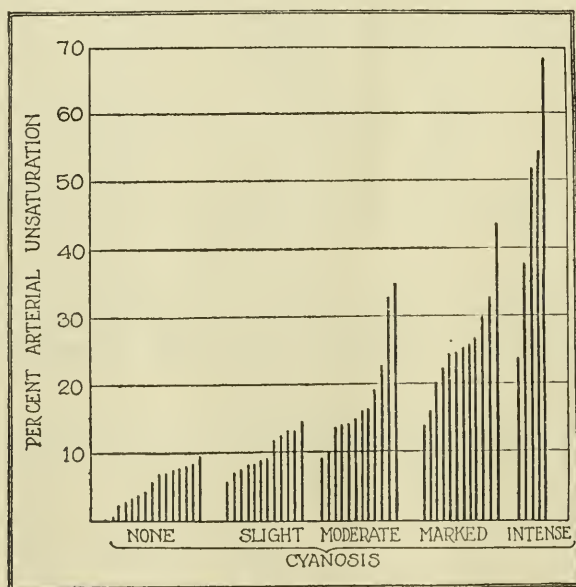
TABLE X.

Arterial and Venous Oxygen Unsaturation Associated with Cyanosis of Varying Degree.

Cyanosis.	No. of observations.	Unsaturation.	
		Mean arterial.	Mean venous.
		per cent	per cent
None.....	18	5.8	23.8
Slight.....	11	11.8	30.4
Moderate.....	10	17.2	41.8
Marked.....	13	26.0	51.2
Intense.....	4	53.2	82.3



TEXT-FIG. 1. Curves showing the relation between arterial and venous unsaturation and degree of cyanosis.

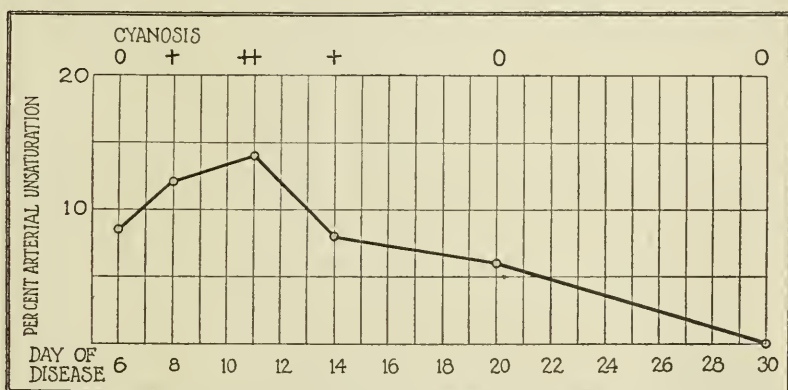


TEXT-FIG. 2. The observations of the per cent arterial unsaturation are plotted in groups according to the degree of cyanosis at the time of observation. The increase of the per cent oxygen unsaturation of the arterial blood with increasing cyanosis is striking.

In Text-fig. 2 the individual values for each observation are plotted in groups according to the degree of cyanosis. Here again the steadily increasing unsaturation with increasing cyanosis is apparent.

Again the relation of cyanosis to blood unsaturation is shown by a study of cases, in which repeated determinations were made on the same patient at different stages of the disease and with varying degrees of cyanosis.

Case 3 (Text-fig. 3) at the first observation (6th day) had no cyanosis, the arterial unsaturation being 8.5 per cent. He became much sicker and had a moderate cyanosis on the 11th day. The arterial unsaturation was then 14.1 per cent. With recovery the cyanosis disappeared, and on the 30th day the arterial unsaturation was 0.0 per cent.



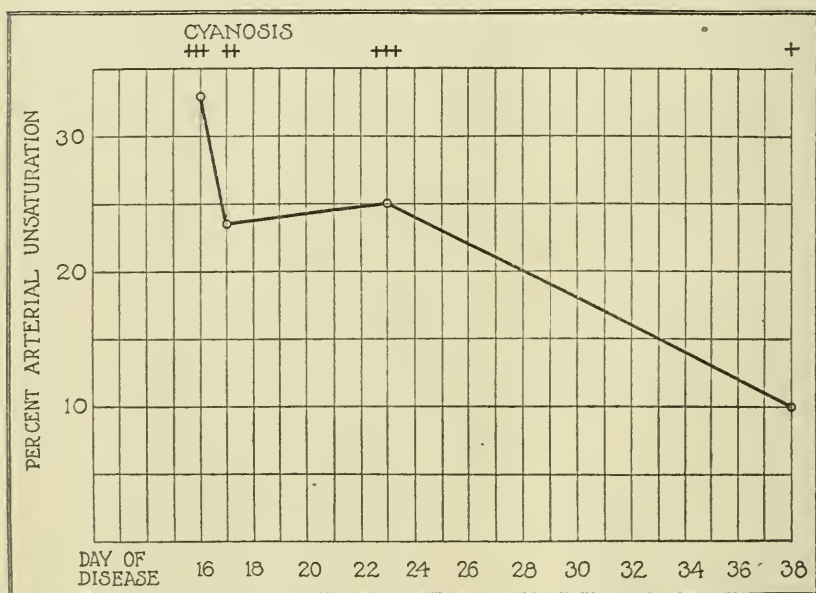
TEXT-FIG. 3. Curve of arterial oxygen unsaturation in Case 3. Note the increase in both cyanosis and arterial unsaturation as the patient became worse. After recovery the cyanosis disappeared and the arterial unsaturation became 0 per cent.

Case 35 (Text-fig. 4) was critically ill and markedly (++++) cyanotic on the 16th day; the arterial unsaturation was 33 per cent. For some time the outlook was desperate and the cyanosis continued associated with an arterial unsaturation of 23.5 and 25.9 per cent on the 17th and 23rd days. Subsequently the patient became much better; the cyanosis diminished until it was slight. The arterial unsaturation was then 10.7 per cent. After complete

recovery (95th day) when no cyanosis was present, the arterial unsaturation was 6.7 per cent.

On the other hand, Case 18 increased the arterial unsaturation from 16.6 per cent to 38.2 per cent as he became worse and the cyanosis increased (Text-fig. 5).

Discussion of Color.—The comparison of the colors of the cyanotic parts with the standard colors in “Répertoire de couleurs”⁹ shows



TEXT-FIG. 4. Curve of arterial oxygen unsaturation in Case 35. There is a gradual decrease of the arterial unsaturation as the patient became better and the cyanosis diminished.

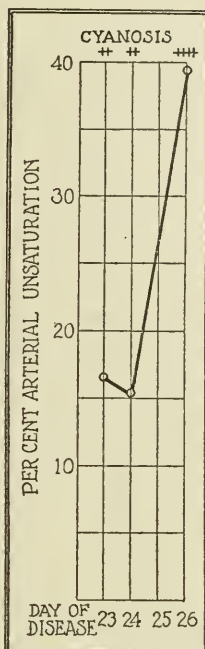
that there is considerable variation in the shades. The basic color is blue, but due to the varying admixtures with red, shades of heliotrope and mauve are frequent, especially in the fingers. On the cheeks a reddish heliotrope is not uncommon, but when the facial cyanosis is diffuse, the color is a leaden or plumbago-blue.

Distribution of Cyanosis.—The most constant and frequent site of the cyanosis was in the end of the fingers, especially under the

⁹ Oberthür, R., Répertoire de couleurs, 1905.

nails. In cases with slight cyanosis a faint but definite bluish tinge could be observed here when no cyanosis could be made out elsewhere.

As the cyanosis became more intense it could next be observed over the entire end of the finger, being more marked on the dorsal aspect and fading gradually toward the first joint and toward the palmar surface.



TEXT-FIG. 5. Curve of arterial oxygen unsaturation in Case 18. The cyanosis increased from ++ to +++ in 3 days, and the arterial unsaturation increased from 16.6 per cent to 38.2 per cent.

Fig. 1 shows the hand in Case 39 with a moderate (++) degree of cyanosis. This corresponded to 14.5 per cent of arterial unsaturation. The cyanosis is confined to the finger-nails and is of the heliotrope shade. In this case there was cyanosis of the lips, nose, and cheeks, but the color was less intense than in the finger-nails.

Fig. 2 shows the hand of a case with intense (++++) cyanosis with an arterial unsaturation of 44.1 per cent. The tips of the

fingers are a darker mauve-blue which is most intense under the nails. On the dorsum the color fades gradually beyond the terminal joint and also toward the palmar aspect.

The cyanosis of the toes is of the same order, but as a rule much less marked.

Second to the fingers the face shows cyanosis. Here it is of different types and distribution. At times even with a high degree of unsaturation, as in Case 34 (Fig. 3) with a marked (+++) degree of cyanosis, there is only a slight, dull, leaden blue, diffusely spread over the face from the forehead to and including the chin. In this case the fingers were darkly cyanotic (+++) and yet the face shows no marked accumulation of color at any one area, and seen alone would not lead one to suspect so high a degree of unsaturation.

Case 31 (Fig. 4) illustrates a second type. Here the 15.3 per cent of arterial unsaturation is accompanied by facial cyanosis in which the color is not diffuse but localized to the areas most frequently involved; namely, nose, chin, lips, ears, and cheeks.

When the cyanosis is slight it may be most marked over the malar bone. Frequently, despite a high arterial unsaturation and deep cyanosis of the fingers, the cyanosis is sharply limited to this area of the face and is of a dull cherry-red-blue at the center surrounded by a fading band of color similar to that seen in the fingers. Fig. 5 is an illustration of this type.

Even with a high arterial unsaturation the chin infrequently shows cyanosis, and the lips and ears, but occasionally. In fact the rarity and lesser degree of cyanosis of the lips in the pneumonia cases is in striking contrast to its frequency in cardiac cases.

Facial cyanosis is characterized also by its variability from day to day and from hour to hour. Often marked changes occurred within an hour. Change of position and coughing produced great changes in the intensity of the facial cyanosis. Therefore, as a measure of the degree of cyanosis from time to time the fingers are the best guide, for here the cyanosis remains most constant.

Total Oxygen Capacity.—A consideration of the oxygen capacity is important to determine whether there is methemoglobin production in pneumonia. In the entire series the total capacity varied from 14.9 to 28.6 cc. In the non-fatal cases (Table XI) there is

no unusually low capacity (except in Case 35) and the mean, 20.0 cc., is slightly lower than the normal mean. In Case 35 there was a drop in 6 days from 19.1 cc. to 15.8 cc. This patient's blood culture was negative and she was desperately ill with marked cyanosis. No examination was made for methemoglobin and the cause of this sudden drop is undetermined.

TABLE XI.
Oxygen Capacity of Non-Fatal Cases.

Case No.	Oxygen capacity per 100 cc. of blood.	Degree of cyanosis.
	cc.	
3	19.9	++
8	18.6	0
10	18.5	0
11	23.6	++
15	20.6	+
17	17.5	+
19	17.2	0
20	20.9	+
23	21.8	+
24	18.6	0
29	20.4	0
35	14.9	+++
36	26.3	+
37	21.8	+
38	19.0	+
39	21.4	++
Mean.....	20.0	

In the fatal cases (Table XII) all had marked cyanosis and should show low capacities if the formation of methemoglobin played an important part in the cyanosis. However, values even slightly below normal were shown only by Case 34. This patient was cyanotic throughout, and during a 3 day period her capacity decreased 0.9 cc. Her blood culture was sterile. With the exception of this the capacities are high and the mean, 23 cc., is higher than the normal.

Again a consideration of the changes in total oxygen capacity (Table XIII) shows that for the fatal cases there was during the illness but slight loss, and many cases show a gain. These changes

are no greater than for the non-fatal cases (Table XIV), particularly those which showed no cyanosis. Case 35 was the only case of the series which showed an unusual fall of capacity (4.2 cc.) associated with marked cyanosis.

It may be said, however, that but four cases, two of which were fatal, had positive blood cultures of pneumococci. Only one (No.

TABLE XII.
Oxygen Capacity of Fatal Cases.

Case No.	Time before death.	Oxygen capacity (gasometric) per 100 cc. of blood.	Degree of cyanosis.
		cc.	
4	7 hrs.	20.3	+++
5	Heart puncture (p.m.).	23.2	++++ during ill- ness.
9	20 min.	24.8	+++
14	2 days.	26.6	+++
16	4 "	24.1	+++
18	3 "	23.7	+++
21	8 hrs.	22.5	+++++
22	12 "	22.3	++
25	1 day.	28.1	+++
30	1 "	22.9	+++
31	6 days.	19.6	++
32	1 day.	26.2	+++
33	1 "	22.2	++
34	1 "	16.2	+++
40		22.3	+++
41		28.6	+++
42		17.8	+++++
Mean		23.0	

22) had an infinite number of colonies per cc. of blood; the others had but a few. This might explain why, unlike Peabody and Harrop, we failed to find occasional cases with greatly reduced oxygen capacity.

It seems unlikely, therefore, that methemoglobin formation plays any important part in the production of the cyanosis here observed, or had any part in the fatal outcome of the sixteen cases.

A striking feature is the unusually high values for the oxygen capacities of some of the very ill or fatal cases. Cases 9, 14, 16, 25, 32, and 41 show this characteristic, but the exact cause of this high capacity is still unknown.

TABLE XIII.
Change in Oxygen Capacity in Fatal Cases.

Case No.	Change in capacity per 100 cc. of blood.	Interval.
	cc.	days
18	-0.2	3
22*	-0.8	2
31	-0.6	5
32	+1.7	1
34	-0.9	3

* Blood culture, *Pneumococcus* Type II colonies ∞ per cc.

TABLE XIV.
Change in Oxygen Capacity in Non-Fatal Cases.

Case No.	Change in capacity per 100 cc. of blood.	Interval.	Remarks.
	cc.	days	
3	-0.0	7	
11	-2.1	16	
15	-1.1	8	
17	-2.0	21	
19	-2.1	9	No cyanosis.
23	+2.0	1	
24	+1.3	19	No cyanosis.
35	-4.2	24	
37	+0.2	1	No cyanosis.

Oxygen Consumption and Heart Failure.

Lundsgaard¹ pointed out that the oxygen consumption, *i.e.* the difference between the arterial and venous oxygen content, increases in cardiac insufficiency. It is also increased by exercise, and presumably by other factors, such as fever, which accelerate the metabolism, unless an equivalent acceleration in circulation occurs.

The oxygen consumption in the series of pneumonia cases presented here is from 0.7 to 10.5 cc. of oxygen per 100 cc. of blood, the average values ranging from 3 to 5 cc., which is the usual range in normal individuals. As a rule, the values for the oxygen consumption in the fatal cases or in the extremely sick were no greater than those in the non-fatal cases, or in the less acutely ill. This would indicate that in the types of pneumonia (chiefly post influenza) represented by our cases the cardiac output does not fall below that normal for the resting organism.

SUMMARY.

1. A simple method for arterial puncture is given which does no permanent injury to the artery. Arterial and venous punctures have been done on 33 cases of pneumonia and five normal subjects, and the blood thus obtained has been studied with reference to the oxygen capacity and arterial and venous unsaturation.

2. In five normal subjects the mean arterial unsaturation was 5 per cent of the total oxygen capacity; the mean venous unsaturation was 26.8 per cent.

3. In the pneumonia cases the arterial oxygen unsaturation varied over a wide range. The arterial unsaturation varied from 0.0 to 68.2 per cent, the venous from 14.4 to 85.5 per cent. In the fatal cases as opposed to the non-fatal cases of pneumonia, the mean arterial oxygen unsaturation was 32 per cent as against 13.9 per cent. As a rule, an arterial unsaturation of over 20 per cent was associated with a fatal outcome. Similarly, the mean venous oxygen unsaturation was 57 per cent in the fatal cases and 36.3 per cent in the non-fatal cases.

4. In five cases in which no cyanosis was observed at any time the mean arterial oxygen unsaturation was 5.4 per cent, the mean venous oxygen unsaturation 31.5 per cent. In cases which showed cyanosis of varying degree during the course of the illness, the mean arterial unsaturation was 24.7 per cent, and the mean venous unsaturation 44.5 per cent. Cases without cyanosis have an arterial unsaturation close to the normal.

5. There is a definite relation between the degree of cyanosis and the per cent of arterial unsaturation. With increasing cyanosis

the arterial unsaturation becomes greater. The venous unsaturation varies similarly.

6. In individual cases with marked cyanosis associated with high arterial unsaturation, the clinical improvement of the patient and the diminution of the cyanosis are accompanied by a similar diminution in the arterial and venous unsaturation. Conversely, an increase of cyanosis is accompanied by an increase in arterial unsaturation.

It is evident that the cyanosis of pneumonia patients is due to the incomplete saturation of venous blood with oxygen in the lungs, and that the various shades of blue observed in the distal parts are caused by an admixture of reduced hemoglobin and oxyhemoglobin in the superficial capillaries.

7. No unusually low total oxygen capacities were observed, even in fatal cases with intense cyanosis. On the contrary, in these cases the total oxygen capacity was unusually high, pointing toward a concentration of the blood. Again in only one case was there any marked fall in the oxygen capacity during the illness. Therefore, methemoglobin formation, in these cases, can hardly have occurred to such an extent as to be an important factor in the production of cyanosis. Of the 33 cases studied, however, only seven were lobar pneumonia, the rest being of types ordinarily unusual, which have accompanied the recent influenza epidemic; and of the seven, not all were in all respects typically lobar. The possibility still remains, therefore, that in typical lobar pneumonia caused by the pneumococcus methemoglobin may play a part in the cyanosis.

8. The oxygen consumption, *i.e.* difference between arterial and venous contents, was within normal limits, indicating that the cardiac output was not diminished in the cases (chiefly post influenza) of pneumonia studied.

EXPLANATION OF PLATES.

PLATE 13.

FIG. 1. Case 39. Arterial unsaturation 14.5 per cent. There is a moderate cyanosis which is confined to the finger-nails. The color is heliotrope.

FIG. 2. Case 25. In this case there is an intense (++++) cyanosis of the fingers associated with an arterial unsaturation of 44.1 per cent. Cyanosis extends as high as the terminal joint and then fades out imperceptibly.

PLATE 14.

FIG. 3. Case 34. Bronchopneumonia; influenza. Arterial unsaturation 29.6 per cent. The entire face is diffusely cyanotic with no especial localization. The color is a leaden blue. The fingers in this case were markedly blue.

FIG. 4. Case 31. Multiple pulmonary abscesses (*Staphylococcus aureus*). Arterial unsaturation 15.3 per cent. There is a marked cyanosis of cheeks, nose, lips, and ears. On the cheek the color is a cherry-red-blue at the center, fading at the periphery to a heliotrope.

PLATE 15.

FIG. 5. Case 35. Bronchopneumonia. Arterial unsaturation 33 per cent. There is a sharply localized area of cyanosis of the cheek. The rest of the face is relatively free. The fingers had a +++ cyanosis.



FIG. 1.



FIG. 2.

(Stadie: Oxygen of the blood in pneumonia.)



FIG. 3.



FIG. 4.

(Stadie: Oxygen of the blood in pneumonia.)



FIG. 5.

(Stadie: Oxygen of the blood in pneumonia.)

THE OXYGEN AND CARBON DIOXIDE CONTENT OF ARTERIAL AND OF VENOUS BLOOD IN NORMAL INDIVIDUALS AND IN PATIENTS WITH ANEMIA AND HEART DISEASE.

By GEORGE A. HARROP, JR., M.D.

*(From the Medical Department of the Johns Hopkins University and Hospital,
Baltimore.)*

(Received for publication, May 14, 1919.)

The oxygen and carbon dioxide content of the blood in man, both in normal and in pathological conditions, has been studied but little, although the close relation of variations in the blood gases to alterations in the respiratory and circulatory mechanisms and to the blood flow has long been recognized. The difficulties of gas analysis upon small quantities of blood and in the technique of collection of samples have rendered the data hard to obtain and the reports in the literature scanty.

With the exception of a few observations by Hürter (1) upon arterial blood, all the data thus far published have been upon venous blood. Such, for example, have been the oxygen and carbon dioxide estimations of Morawitz and Röhmer (2) upon patients with anemia, those of Peabody (3) upon patients with lobar pneumonia, and those of Means and Newburgh (4) and of Lundsgaard (5) upon patients with cardiac disease.

Lundsgaard (6) has reported a series of thirty-eight determinations of the oxygen content of venous arm blood on twenty normal individuals. Hürter has reported four determinations of the oxygen and carbon dioxide content of normal human arterial blood. He found the arterial oxygen saturation to be between 93 and 100 per cent of the maximum capacity, which agrees with the values calculated from the dissociation curve for oxyhemoglobin.

The study of the arterial blood begun in 1912 by Hürter's analyses failed to develop further until 1918, when Stadie (7), continuing in the Hospital of The Rockefeller Institute the work on blood gases begun there by Lundsgaard and

Van Slyke (8), successfully undertook routine analyses of the arterial blood in patients with pneumonia.¹

The determinations of Lundsgaard, which gave the most complete data available upon cardiac disease, were done with the recently devised blood gas apparatus of Van Slyke (8). The simplicity, accuracy, and speed attainable with this method render it a most satisfactory procedure. It has been employed in all the determinations here reported.

The collection of blood samples has been done in some instances by the method described by Lundsgaard for venous blood, and in others by the procedure recently described (9). Briefly, venous blood is collected without stasis from a vein at the bend of the elbow, and arterial blood from the radial artery, by means of a 20 cc. Luer syringe, to which is attached a sharp, short beveled needle.² The end of the syringe is rendered free from air by filling with 1 or 2 cc. of sterile paraffin oil, which is made to wet the barrel throughout its length. The technique of artery puncture is acquired easily, and when properly done causes little more discomfort to the patient than an ordinary venipuncture. The hand is held in the position recommended by Hürter (1), an assistant steadying it in place. After one has acquired a little practice, it is seldom necessary to use any local anesthesia. The only serious difficulty which may be met with is hematoma formation, which may take place from the opening in the artery. If proper steps to prevent it are neglected, a considerable extravasation of blood may occur and cause great pain and inconvenience. A small pressure bandage is applied tightly over the point of puncture immediately on withdrawal of the needle, and retained in place for at least an hour. Over 125 radial artery punctures and about 10 brachial artery punctures have now been done in this clinic, without any untoward effects.

Blood Gas Content in Normal Resting Individuals.

In Table I are given the results of oxygen determinations upon the arterial and venous blood of fifteen individuals with normal respiratory and circulatory apparatus, upon ten of whom simultaneous carbon dioxide analyses also were made. The artery punctures

¹ I am indebted to Dr. William C. Stadie for communicating to me his experience with the arterial puncture shortly after he had satisfied himself that it could be done without danger. In consequence of this, the present work on cardiac patients could be prosecuted simultaneously with Dr. Stadie's studies on pneumonia.

² The size used is No. 19 or 20 gauge, and the length $1\frac{1}{2}$ to 2 inches. Care is taken that the needles are clean and very sharp.

TABLE I.

Arterial and Venous Oxygen and Carbon Dioxide Determinations upon Individuals with Normal Heart and Lung Findings.

Individual No.	Oxygen capacity (A).	Oxygen content of arterial blood (B).	Percentage saturation of arterial blood $\left(\frac{B}{A} \times 100\right)$.	Arterial oxygen unsaturation (A-B).	Oxygen content of venous blood (C).	Oxygen consumption (B-C).	Carbon dioxide content of arterial blood.	Carbon dioxide content of venous blood.	Temperature.	Pulse.	Respirations.	Blood pressure.	Remarks.
	vol. per cent	vol. per cent		vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	°F.			mm.	
1	23.74	23.04	97.5	0.70	17.61	6.43	51.76	57.15	98.6	78	20	$\frac{125}{80}$	
2	17.23	17.19	100.0	None.	14.62	2.57	54.69	56.71	98.6	86	22	$\frac{132}{84}$	
3	16.26	15.31	94.3	0.95	10.52	4.79	52.89	55.88	101.4	88	22	$\frac{120}{80}$	
4	20.60	19.83	96.3	0.77	13.46	6.37	46.46	51.70	98.4	84	20	$\frac{115}{78}$	
5	18.69	17.75	95.1	0.94	15.09	2.66	44.84	48.27	99.0	84	22	$\frac{130}{84}$	
6	20.60	19.79	96.0	0.81	12.74	7.05	49.67	54.56	98.8	80	20	$\frac{128}{78}$	
7	14.42	13.89	96.3	0.53	10.77	3.12	48.13	52.18	98.8	82	20	$\frac{120}{80}$	
8	24.67	24.08	97.6	0.59	15.81	8.27	50.55	58.74	98.6	84	22	$\frac{130}{78}$	
9	18.91	17.87	94.4	1.04	13.94	3.93	53.31	60.43	98.8	86	20	$\frac{126}{85}$	
10	21.23	21.01	99.0	0.22	13.08	7.93	44.58	50.90	98.6	82	20	$\frac{120}{80}$	
11	22.02	20.94	94.9	1.08	15.04	5.90			98.4	80	24	$\frac{130}{85}$	
12	21.46	21.23	98.9	0.23	14.66	6.57			98.6	78	22	$\frac{150}{95}$	
13	20.85	19.78	94.8	1.07	14.09	5.69			98.8	78	20	$\frac{128}{80}$	
14	22.08	21.00	95.0	1.08	13.98	7.02			98.6	76	20	$\frac{132}{78}$	
15	19.76	18.92	95.8	0.84	15.17	3.75			98.6	84	22	$\frac{140}{80}$	Right radial artery.
	19.58	19.10	97.6	0.48					98.6	84	22		Left brachial artery.
	19.82	19.24	97.2	0.58					98.6	84	22		Left radial artery.

for the three determinations made on the last case in the table, No. 15, upon the right radial artery, the left radial artery, and the left brachial artery, in the order named, were all done in the space of about 12 minutes and were made with the purpose of furnishing some experimental evidence of variations, if any exist, in the blood gas content of differently placed arteries.

The term oxygen unsaturation was introduced by Lundsgaard in his studies upon the venous blood, to indicate the difference in volumes per cent between oxygen content and oxygen capacity. It is used here in the same sense.

The term oxygen consumption, used by various writers, is applied here to the difference between the oxygen content of the arterial and of the venous blood in volumes per cent.

All the blood specimens for the determinations reported in this paper were uniformly collected in the following way: The artery puncture was first done and was immediately followed by the vein puncture. Both were done within a space of 3 to 5 minutes. The pulse and respirations were then counted. The blood pressure was that recorded on the history chart as the day's reading. All the individuals were bed patients unless otherwise indicated, and the specimens were taken at least 2 hours after the preceding meal.

The determinations were done immediately after collection, in nearly all instances those for carbon dioxide by one worker and those for oxygen by the other, simultaneously. When one person has done both series, those for carbon dioxide were made first because of the tendency of the carbon dioxide to diffuse out into the protecting paraffin oil layer.

The oxygen capacity readings were in all cases checked by colorimetric determinations by the Palmer method. However, the readings taken were the average of those made directly with the blood gas apparatus.

The factors producing variation in the oxygen content of arterial blood, aside from the obvious change in the oxygen-carrying capacity of the blood due to the varying content in hemoglobin, are chiefly those due to alterations in the lung ventilation (the alveolar oxygen tension), to pathological changes in the lung tissue, and to variations due to the rate of the blood flow, as found in normal individuals, for example, between the periods of rest and exercise.

It seems unlikely that variation in the phases of normal respiration in man usually effects as large changes as have been credited to it (1 to 2 volumes per cent).

The following observation upon the oxygen content of the arterial blood in an individual with Cheyne-Stokes respirations (the period of apnea was of 45 seconds duration), certainly producing a much greater interference with the blood gas exchange in the lungs than is caused by the normal variation in the phases of respiration, offers some evidence on this point.

Specimen.	Oxygen capacity.	Oxygen content of arterial blood.	Percentage saturation of arterial blood.
	vol. per cent	vol. per cent	
At height of period of dyspnea.....	24.61	24.51	100 (right radial artery).
" middle " " " apnea.....		22.00	89.4 (left " ").

In spite of the marked disturbance in the breathing, the difference in the arterial oxygen content between the two phases amounted to but 2.51 volumes per cent. It was not possible to make a satisfactory collection of a venous sample, owing to the much slower rate of flow of venous blood, collected without stasis.

Little variation can take place in the gas content of the blood in the radial artery, as compared with that leaving the left heart, and there can be no changes in the particular sample due to variations in metabolism or local variations in the blood flow, which are not common to the arterial blood as a whole, as it exists in the larger vessels. The close agreement of the gas content in blood removed from various arteries (Case 15, Table I) furnishes experimental evidence on this point. The oxygen content of normal venous blood varies widely,³ due to a combination of many factors. The most important of these are the variations in the gas content of the arterial blood and the local variations in the blood flow. The influence of the first factor is well illustrated in the determinations on patients with severe anemia, given in Table II.

The very low oxygen content of the venous blood in these patients with the resulting extremely low pressure-head in the capillaries is an indication of the ability of the tissues to take up oxygen from the

³ According to Lundsgaard between 18 and 9.5 volumes per cent.

TABLE II.

Case No.	Date.	Oxygen capacity.	Oxygen content of arterial blood.	Arterial oxygen unsaturation.	Oxygen content of venous blood.	Oxygen consumption.	Diagnosis.
	1919	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	
1	Mar. 27	4.12	3.31	0.81	1.55	1.76	Acute lymphatic leucemia.
2	Apr. 1	4.17	3.83	0.34	1.31	2.52	Uremia. Bleeding from gums and vagina.
3	" 15	3.68	3.50	0.18	0.87	2.63	Pernicious anemia.

blood over a wide range of oxygen pressures. Even at these low levels the oxygen consumption remained within practically normal limits.

The effect upon the blood gases of alteration in the blood flow may be shown by a comparison of the analyses of blood samples taken during periods of rest and after exercise.

The experiment shown in Table III was made upon a normal individual, white, male, age 29 years. Two determinations were made (*a*) after lying down quietly for 30 minutes, and (*b*) immediately after 15 minutes of brisk exercise, consisting of arm and trunk movements, and vigorous hopping about the room until quite dyspneic. The pulse and respiration readings were then made while the arterial blood was being withdrawn. The collection of the venous blood sample was then made.

TABLE III.

Condition.	Oxygen capacity.	Oxygen content of arterial blood.	Percentage saturation of arterial blood.	Oxygen content of venous blood.	Oxygen consumption.	Carbon dioxide content of arterial blood.	Carbon dioxide content of venous blood.	Temperature.	Pulse.	Respirations.
	vol. per cent	vol. per cent		vol. per cent	vol. per cent	vol. per cent	vol. per cent	°f.		
Resting.....	22.04	21.09	95.6	15.08	6.01	53.33	56.90	98.6	86	18
After exercise.....	22.41	19.19	85.5	12.93	6.26	32.25	41.14		140	30

I hope to report at a later date a study in detail of the effects of exercise. Presumably later changes of a compensating nature may occur, in the direction of a restoration of the normal arterial oxygen saturation possibly by increased secretion of oxygen by the lung epithelium, or by regulation of the oxygen consumption in the tissues, which may explain the phenomenon of acquiring one's second wind.

Blood Gases in Cardiac Disease.

The methods just described have been applied to the study of the gas content of the blood in persons suffering from cardiac disease.⁴ A considerable number of determinations has been made upon the arterial and venous blood of patients, clinically compensated, and without cardiac arrhythmias, at rest in bed. No noteworthy deviation from the values given above for normal persons has been found and it seems of no practical value to publish these results. In Table IV are given the results of determinations upon nine persons with decompensated circulatory disturbances. The clinical data upon these patients are given below.

Case 1.—J., white, male, machinist; age 50 years.

Diagnosis.—Emphysema; arteriosclerosis; hypertension; chronic myocarditis.

Previous History and Symptoms.—Shortness of breath on exertion for many years; four periods of decompensation in the past 6 years. Present attack has lasted for 3 months and has been particularly bad for the past 5 days. No history of syphilis or rheumatic fever.

Physical Examination (Apr. 25, 1919).—Slight cyanosis of finger-tips. Moderate orthopnea. Moderate engorgement of neck veins. Cardiac borders are hard to define accurately on account of the marked grade of emphysema present, but the dullness is considerably increased both to left and to right; no murmurs; no arrhythmia. Second sounds at aortic and pulmonic areas of about equal intensity. Dullness at right lung base, with many moist râles at either base. No ascites. Pitting edema of lower legs. Wassermann reaction negative.

Apr. 28. Patient has shown rapid response to treatment. Practically no respiratory difficulty in bed. Lungs clear. Edema almost gone. Pulse regular except for one extrasystole with each 10 to 15 heart beats.

May 5. No longer any signs of myocardial insufficiency at rest in bed. Has severe attacks of cardiac dyspnea at night.

⁴ The samples were collected in these cases, as in all herein reported, upon bed patients while recumbent. The only exception was in the case of badly decompensated patients with a marked grade of orthopnea, in which instances the blood was taken from the patients when they were in a sitting posture.

4	60	Mar. 29	16.78	13.90	82.8	2.88	10.84	3.06	41.40	44.19	97.8	80	32	$\frac{154}{100}$	Chronic myocarditis; auricular fibrillation.
		Apr. 5	16.61	15.23	91.7	1.38	9.58	5.65	43.51	46.48	98.4	48	24	$\frac{140}{98}$	
5	30	Feb. 16	19.87	17.70	89.1	2.17	6.30	11.40	38.88	41.76	98.8	96	24	$\frac{110}{68}$	Chronic rheumatic endocarditis; mitral stenosis and insufficiency; auricular fibrillation.
		" 24	19.68	18.15	92.3	1.53	10.42	7.73	42.68	47.05	98.7	92	28	$\frac{115}{80}$	
		Mar. 4	19.74	17.68	89.5	2.06	8.31	9.37			98.4	96	28	$\frac{112}{78}$	
		" 18	19.62	17.90	89.8	1.72	13.07	4.83	43.80	47.61	98.6	88	26	$\frac{116}{80}$	
6	38	Apr. 9	18.68	16.46	88.2	2.22	12.94	3.52			98.6	140	36	$\frac{110}{80}$	Mitral and aortic insufficiency; adherent pericardium; bilateral hydrothorax.
		" 10	19.09	15.56	81.5	3.53	8.49	7.07			99.0	170	48	$\frac{103}{75}$	
7	39	Jan. 2	18.85	16.48	87.4	2.37	7.29	9.19	38.65	43.21	98.4	100	36	135	Syphilis of aorta; aortic insufficiency.
		" 10	22.92	20.52	85.9	2.40	16.10	4.42	42.87	46.48	98.6	98	26	145	
		" 20	22.48	21.09	93.7	1.39	15.42	5.67	43.62	49.01	98.4	78	20	145	
8	33	Apr. 29	20.74	19.31	93.2	1.43	11.13	8.18	31.42	37.53	98.2	96	30	128	Aortic insufficiency.
		May 1	22.50	21.30	94.7	1.20	11.64	9.66	42.80	45.70	98.0	84	24	125	
		" 6	23.25	22.49	96.8	0.76	17.55	4.94	41.47	46.52	98.6	88	24	125	
9	42	Jan. 30	17.97	16.71	93.0	1.26	6.64	10.07			97.4	100	28	140	"
		Feb. 24	16.33	15.96	97.7	0.37	9.82	6.14			98.2	90	22	148	"

It will be noticed that the percentage saturation of the arterial blood with oxygen at the time of admission was much lower than normal. The chest showed well marked emphysema, and signs of congestion in the lungs were apparent. With the clearing up of this congestion and the return to compensation the arterial oxygen saturation quickly returned to normal. At the same time the oxygen content of the venous blood rose and the oxygen consumption correspondingly became less.

Case 2.—L., negro, male, laborer; age 57 years.

Diagnosis.—Emphysema; hypertension; chronic myocarditis.

Previous History and Symptoms.—Recurrent periods of decompensation for several years. No history of syphilis or rheumatic fever.

Physical Examination (Feb. 24, 1919).—Moderate dyspnea. No definite cyanosis. Huge heart, with apex in midaxilla; no murmurs; rate regular except for an occasional extrasystole. Moderate dilatation of neck veins. Slight edema of legs. Numerous moist râles and dullness at the lung bases. Liver edge well below the costal margin and tender. Wassermann reaction negative.

Feb. 29. Patient has responded well to treatment. The lungs are clear. No liver tenderness. Practically no respiratory distress. Still some extrasystoles; no cyanosis; no edema.

Mar. 15. Condition is not changed. The extrasystoles persist.

The rapid improvement in the symptoms of decompensation in this case, which responded to treatment almost as rapidly as Case 1, produced coincident improvement in the oxygenation of the arterial blood with the clearing up of the signs of lung congestion.

Case 3.—S., white, male, occupation not given; age 50 years.

Diagnosis.—Chronic bronchitis; emphysema; chronic myocarditis.

Previous History and Symptoms.—Symptoms of emphysema and of chronic bronchitis for many years. Dyspnea on exertion for 25 years. Winter cough for past 10 years. Gradual onset more recently of the cardiac symptoms.

Physical Examination (Feb. 21, 1919).—Signs of right-sided cardiac hypertrophy, with secondary myocardial insufficiency. Voluminous, hyperresonant lungs; bases descend very little, and there are numerous fine râles. Heart borders overlapped by lungs, but distinctly enlarged to the right; the sounds are faint, almost inaudible, but seem clear. Slight edema of legs; no liver tenderness; no particular engorgement of the superficial veins. Marked cyanosis of lips, face, and finger-tips, out of proportion to the involvement of respiration. Expectoration of purulent sputum. Wassermann reaction negative.

Feb. 26. Condition is greatly improved, but there are still many râles at the lung bases, and there is considerable cyanosis.

Mar. 4. Condition further improved. Râles still present at bases. Cyanosis still present.

In this case there was distinctly evident a superimposed lung factor, long standing chronic bronchitis and emphysema, which produced a marked effect on the normal oxygenation of the blood. Even at discharge the percentage saturation of the arterial blood with oxygen was below the low normal value, and the patient was distinctly cyanotic. This chronic state of cyanosis had been present for years, although much aggravated at the time of his cardiac break. The oxygen content of the venous blood was about normal over the period of the observations.

Case 4.—M., negro, male, laborer; age 60 years.

Diagnosis.—Chronic myocarditis; auricular fibrillation.

Previous History and Symptoms.—Onset of disorder in Nov., 1918, with cough and shortness of breath. Swelling of legs 3 days ago.

Physical Examination (Mar. 29, 1919).—Marked orthopnea. Huge heart, enlarged downward and to the left; second pulmonic sound accentuated; no murmurs; rate totally irregular. Pulse deficit about 10 beats per minute. Many moist râles at either base, especially at the right. No particular cyanosis. Slight tenderness at right costal margin. Pitting edema of legs, extending up to the knees. Neck veins somewhat distended. Wassermann reaction negative.

Apr. 5. Condition much improved, although distinct orthopnea is still present. Pulse very slow, 48 per minute, all the beats now coming through to the wrist. No cyanosis or liver tenderness. Edema rapidly clearing up. Râles at lung bases have practically cleared up. Electrocardiogram indicates auricular fibrillation.

Case 5.—C., negro, female; age 30 years.

Diagnosis.—Mitral stenosis and insufficiency; auricular fibrillation.

Previous History and Symptoms.—Rheumatic fever 3 years ago. No history of syphilis. Cardiac symptoms, especially shortness of breath and occasional precordial pain, have been present during the past 10 months.

Physical Examination (Feb. 16, 1919).—Marked orthopnea and dyspnea. Totally irregular heart; about 15 beats per minute fail to come through to the wrist. Râles and dullness at the lung bases. Only moderate cyanosis of lips and finger-tips. Marked edema of legs and hands. Tender, palpable liver; certainly some fluid in abdomen. Systolic and diastolic murmurs at apex. Snapping first sound. Accentuated second pulmonic sound. Wassermann reaction negative.

Feb. 24. Rather slow response to digitalis. Condition, however, seems improved. Signs of congestion somewhat less marked.

Mar. 4. Slow but distinct improvement. Edema gone. Râles and dullness no longer present at lung bases.

Mar. 18. Seems practically compensated clinically. Heart rate 88, all beats coming through to the wrist. Lungs clear of râles.

The percentage saturation of the arterial blood in this patient tended to remain definitely below the normal figure, even after compensation was restored. As far as could be determined clinically, the lung bases were clear. There was nothing to suggest adherent pericardium; the heart, although large, was clearly movable with change in position. Nevertheless, the patient gave every indication of having had long standing cardiac disease, despite her rather short and possibly inexact history. She exhibited well marked mitral facies and clubbing of the fingers. The x-ray of the chest was reported as showing considerable infiltration, particularly of the lower parts of both lungs. The figures for the oxygen consumption tended to show the same erratic irregularities to which, in auricular fibrillation, Lundsgaard (5) has already drawn attention.

Case 6.—N., white, male, sailor; age 38 years.

Diagnosis (Autopsy).—Mitral and aortic insufficiency; adherent pericardium; bilateral hydrothorax.

Previous History and Symptoms.—Patient was admitted very ill. Said to have been sick 2 weeks with shortness of breath. Expectoration of blood-tinged sputum for several days.

Physical Examination (Apr. 9, 1919).—Marked dyspnea and cyanosis of lips, face, and fingers. Rapid but regular pulse. Lungs practically clear. Marked retrosternal dullness and huge heart. Moderate engorgement of neck vessels. No edema. Soft, pulsating liver, edge at umbilicus. Systolic and diastolic murmurs at apex and soft aortic diastolic murmur to left of sternum. Wassermann reaction negative.

Apr. 10. Condition decidedly worse, with delirium and marked cyanosis. No fever. Pulse weak and rapid. Heart and lung signs unchanged, except that signs of pulmonary edema developed rapidly shortly after the blood specimens were taken. Patient died about 16 hours later.

Autopsy.—Huge heart to which the pericardium was everywhere adherent. The lungs were clear except for the compression due to a bilateral hydrothorax of 1,500 cc. on either side. The liver was enlarged.

This case is of particular interest in that the patient showed cyanosis of extreme degree, and it was thought possible that pneumonia was present as well as the cardiac disease. This opinion proved incorrect, however, and the lung changes were all found to be secondary to the cardiac deficiency.

All the evidence pointed to a rapid appearance of serious respiratory embarrassment on account of the acute decompensation and the resulting accumulation of pleural fluid. It seems not unlikely that the time element plays a certain part in some of these cases, and that in this particular one, had the respiratory apparatus had time to adjust itself to the new conditions, a more efficient oxygen saturation of the arterial blood would have been later effected, even if the cause of the lung compression remained.

Case 7.—S., negro, male, laborer; age 35 years.

Diagnosis.—Syphilis of aorta; aortic insufficiency.

Previous History and Symptoms.—Indefinite history of shortness of breath about 1 year ago. This improved but again became serious about 6 months ago, since which time he has worked but little. Swelling of the legs and orthopnea for past 3 months. No history of rheumatism.

Physical Examination (Jan. 2, 1919).—Orthopnea; swelling of neck veins; massive pitting edema of legs; slight cyanosis. Huge heart with loud aortic diastolic murmur; rate regular. Palm's breadth of marked dullness at right base; many moist, bubbling râles at either base. Wassermann reaction positive.

Jan. 10. Condition has improved with rest and the usual cardiac treatment. Nevertheless, the pulse is 98 and there is still distinct orthopnea. Râles still persist at either base and considerable dullness is still present at the right base.

Jan. 20. Patient has gradually improved and there is no longer any respiratory distress. Pulse 78. Lung bases practically clear. No edema and no liver tenderness.

Case 8.—H., negro, male, laborer; age 33 years.

Diagnosis.—Aortic insufficiency.

Previous History and Symptoms.—Shortness of breath for 2 months. Swelling of legs noticed 2 days ago. History of syphilis. Some bloody sputum expectorated the day previous to admission.

Physical Examination (Apr. 29, 1919).—Moderate dyspnea and slight orthopnea. No cyanosis or dilatation of superficial veins. Lungs clear except for a few crackles at the bases; no dullness. Huge heart, with apex in the anterior axillary line; increased retrosternal dullness; musical aortic diastolic murmur along left sternal border and in aortic area. Collapsing pulse, regular. Very slight liver tenderness. Moderate edema of lower legs. Wassermann reaction positive.

May 1. Some bloody expectoration yesterday. Considered likely that lung infarcts have been produced, but no pain in chest is complained of, and lungs show no new findings. Breathing improved. Edema less.

May 5. Compensation is now almost regained. Lungs are clear. Pulse much slower and regular. No cyanosis; no edema; no liver tenderness.

Case 9.—R., negro, male, laborer; age 42 years.

Diagnosis.—Syphilis of aorta; aortic insufficiency.

Previous History and Symptoms.—Has had shortness of breath for several months and swelling of legs for 2 weeks. Hard chancre 4 years ago.

Physical Examination (Jan. 30, 1919).—Orthopnea and dyspnea are marked. No cyanosis. Lungs are practically clear. Wide retrosternal dullness. Huge heart with the apex impulse in midaxilla, with signs of aortic insufficiency. Typical pulse, regular in rhythm. Slight edema of legs. Tenderness at right costal margin. Some engorgement of neck vessels. Wassermann reaction positive.

Feb. 24. Compensation is quite regained. Lungs clear.

The last three patients (Cases 7, 8, and 9) exhibited the same type of cardiac lesion with varying degrees of decompensation. Of these, Case 7 was suffering from a much more severe break than the other two, and his recovery under cardiac therapy (milk diet, restricted fluids, digitalis, and rest) was much slower. His lower chest was filled with moist râles at the time of the first examination, and it seemed likely that there was a certain amount of pleural effusion at the right base.

Although it was considered probable at the time (May 1) that Patient H. (Case 8) had had a pulmonary infarct because of his bloody sputum, no other signs appeared, there was no abnormality found in the content of blood gases, and his return to compensation was prompt.

The condition of Patient R. (Case 9) at the time of the first blood oxygen examination, on the 4th day following admission, was not serious, except for his rather marked orthopnea and enlarged liver. The lungs showed no outspoken evidence of congestion. The oxygen consumption at this examination was high—presumably there was rather marked increase in the metabolism with the severe respiratory exertion, but the percentage saturation of the arterial blood was about normal. At the time of the second examination (February 24), when the findings were normal, compensation had been entirely regained.

CONCLUSION.

An examination of the data presented in these cases of cardiac disease indicates the importance of the effect of the primary condition of the lungs upon the oxygen saturation of the arterial blood during periods of decompensation. Emphysema and chronic inflammatory processes appear to aggravate greatly the effect of the passive congestion due to the cardiac insufficiency alone.

It is desired to take up at another time the effects produced by disturbances to the passage of air through the trachea and its branches. Sufficient data have accumulated, however, to warrant the expression of an opinion that the time element is of importance. Sudden mechanical disturbances to breathing produce abnormal changes in the concentration of the blood gases. These disturbances, after a period, even if unrelieved, are in large part compensated.

Because of the striking variation in the color of blood due to changes in its oxyhemoglobin content, it appears possible to relate the degree of cyanosis in individuals to the extent of oxygen unsaturation. The determining factor in the production of cyanosis must be the color of the blood in the superficial capillaries and veins, although the amount of skin pigmentation no doubt influences greatly its appreciation clinically. The cause of the phenomenon probably varies. One type is produced where incomplete saturation of the arterial blood is at fault, due to disturbance in the normal gas exchange in the pulmonary alveoli, as in pneumonia. In many cases of heart disease it may be a combination of both arterial and primarily venous unsaturation; in others it is the venous unsaturation due to stasis which is chiefly at fault. It is probable that in other instances there may be disturbance neither in the pulmonary exchange nor in the blood flow in the larger venous vessels, but that constriction of peripheral vessels due to local stimuli, with consequent local slowing of the blood flow and necessarily increased oxygen consumption, produces a type of "capillary" cyanosis, such as is, for example, strikingly seen in bathers exposed to the cold air when wet.

SUMMARY.

1. The oxygen content of venous and of arterial blood from fifteen essentially normal individuals at rest in bed has been determined.

2. The percentage saturation of the arterial blood has varied between 100 and 94.3. The average is 95.5 per cent.

3. The oxygen consumption has varied between 2.6 and 8.3 volumes per cent.

4. The oxygen content and the percentage saturation of arterial blood taken at close intervals from three different peripheral arteries of a normal individual have shown values agreeing within the limits of error. Analyses of the blood gases of a normal individual, at rest and after exercise, have shown a lowering of the percentage oxygen saturation of the arterial blood and a diminished carbon dioxide content after exercise.

5. In three persons with severe anemia the saturation of the arterial blood has not differed from the normal. Very low absolute values were found for the oxygen content of the venous blood, but the normal oxygen consumption has been maintained.

6. The carbon dioxide content of the arterial blood from ten normal individuals has varied between 54.7 and 44.6 volumes per cent. That of the venous blood has varied between 60.4 and 48.3 volumes per cent.

7. No deviations from the normal values for oxygen and carbon dioxide were found in venous and arterial blood from cardiac patients without arrhythmias, well compensated, and at rest in bed.

8. A series of determinations has been made upon nine cardiac patients with varying degrees of decompensation. The percentage oxygen saturation of the arterial blood on admission was abnormally low in seven of these cases. With the return to compensation and with the clearing up of pulmonary symptoms, the percentage saturation of the arterial blood returned to normal in four of them.

9. In a case of long standing mitral endocarditis with auricular fibrillation it remained low over a period of 1 month of observation.

10. In a case of chronic myocarditis secondary to emphysema and chronic bronchitis, it remained low over the period of observation.

11. Normal values for the percentage saturation of the arterial blood were found in two individuals with decompensated aortic disease but without physical signs of extensive pulmonary involvement.

12. The oxygen consumption tended to be high in individuals with cardiac disease during the periods of marked decompensation and to be lower as compensation was regained.

13. The data presented indicate that at least in many circulatory diseases during decompensation, particularly when there are physical signs of pulmonary congestion, there is a disturbance of the pulmonary exchange, as indicated by the lowering of the percentage saturation of the arterial blood with oxygen.

I desire to acknowledge the assistance of Miss Ethel M. Benedict, Assistant in Medicine, who performed many of the gas analyses used in this paper.

BIBLIOGRAPHY.

1. Hürter, *Deutsch. Arch. klin. Med.*, 1912, cviii, 1.
2. Morawitz, P., and Röhmmer, W., *Deutsch. Arch. klin. Med.*, 1908, xciv, 529.
3. Peabody, F. W., *J. Exp. Med.*, 1912, xvi, 701.
4. Means, J. H., and Newburgh, L. H., *Tr. Assn. Am. Phys.*, 1915, xxx, 51.
5. Lundsgaard, C., *J. Exp. Med.*, 1918, xxvii, 179.
6. Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxiii, 133.
7. Stadie, W. C., *J. Exp. Med.*, 1919, xxx, 215.
8. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347; 1918, xxxiii, 127.
9. Harrop, G. A., Jr., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 10.

STUDIES ON CYANOSIS.

I. PRIMARY CAUSES OF CYANOSIS.

By CHRISTEN LUNDGAARD, M.D.

(From the Medical Clinic of the University of Copenhagen, Copenhagen, Denmark.)

(Received for publication, May 23, 1919.)

INTRODUCTION.

In medical terminology cyanosis indicates a blue or bluish color of the skin of the mucous membranes, and of other organs usually not visible (the kidneys, the spleen, etc.). Cyanosis may be general or local. In the latter instances, it is usually found on the lips, nose, ears, fingers, and toes (acrocyanosis). It may be met with in various pathological conditions, and is found especially in patients suffering from diseases of the heart, lungs, and blood. Since it is a striking sign, cyanosis has been known since the early history of medicine, and has attracted much attention.

Various theories have been put forward about its pathogenesis, but none has been generally accepted. Cyanosis is an important symptom in the differential diagnosis of various diseases, and it has always been considered important for the prognosis of the diseases in which it is encountered. Since it is usually considered a harmful condition, special therapeutic procedures (venesection, oxygen inhalations) have often been used against it.

HISTORICAL.

De Senac (1749) (1) gave the first explanation of the pathogenesis of cyanosis. He considered it the result of an admixture of arterial and venous blood due to an abnormal communication between the two sides of the heart, which he found in autopsy. In 1761 Morgagni (2) showed that cyanosis might be combined with pulmonary stenosis. He explained the cyanosis by stasis caused by the pulmonary stenosis. Grancher (3) found a distention of the capillaries and the smallest arteries in patients suffering from (congenital) cyanosis, and considered this distention as well as the cyanosis as caused by stasis. Later Knapp (4) detected the alterations of the retinal vessels in patients with congenital cyanosis. This, together with experiments on animals by Panum (5), Dareste (6),

Stockard (7), Bardeen (8), Loeb (9), and others, resulted in giving the capillary anatomical changes more prominent and causal relation to cyanosis, which in some instances was considered due to anatomical malformation of the small vessels. Abnormalities in the blood itself have also been held responsible for the production of cyanosis. Since Krehl (10) found polycythemia in patients with congenital cyanosis and Vaquez (11) (1892) described the first case of polycythemia, an abnormally high number of red blood corpuscles has been considered a cause of cyanosis.

Alterations in the blood gases have also been considered the cause of cyanosis, and in spite of lack of experimental confirmation, this idea has never been given up. Some had the opinion that an accumulation of carbon dioxide in the blood, others that too small an amount of oxygen caused the cyanotic skin color. Also the amount of reduced hemoglobin has been considered the cause. Von Bamberger (12) early put this idea forward in his monograph on heart diseases. Since then, several others have used the same explanation, and it is usually considered one of the most important causes of cyanosis. No experimental proof has, however, been established. In a previous paper by the writer (13) it was pointed out that the results of a series of determinations of venous oxygen on decompensated heart patients seemed to show that there existed a close relation between cyanosis and the amount of reduced hemoglobin (called oxygen unsaturation) of the venous blood. Later Langstroth (14) confirmed this in two heart patients suffering from cyanosis. But the problem has never been submitted to a systematic investigation.

Present State of the Problem of the Cause of Cyanosis.

The literature shows that some confusion prevails, primary and secondary causes being intermixed. By primary causes I mean changes of the blood itself; by secondary causes I mean pathological disturbances, which may be more or less localized. Such intermixture of primary and secondary causes results in confusion such as we would have if we defined anemia as a condition caused by different factors; for instance, decreased hemoglobin, bleeding gastric ulcer, toxins, and so forth.

Primary Causes of Cyanosis.—Of what we may term primary causes, three are held especially responsible: (1) increased carbon dioxide content of the venous blood; (2) decreased oxygen content of the venous blood; and (3) increased oxygen unsaturation of the venous blood.¹

¹ The term oxygen unsaturation has previously (15) been defined as "the difference between the venous oxygen and the total oxygen-combining power of the hemoglobin."

The chief aim of the present work has been to investigate whether or not any of these alterations of the blood really accounts for the cyanosis; *i.e.*, is the primary cause. For that purpose the cyanosis and the blood findings on a series of cyanotic and non-cyanotic individuals have been compared.

Technique.

The cyanosis is simply estimated (always in full daylight) and its degree indicated by plus signs. Three plus signs mean a very dark blue color. It has always been general (skin and mucous membranes) but never quite equally distributed. One plus indicates a just visible bluish color, usually but not always localized to the peripheral parts (acrocyanosis). Two pluses indicate an intermediate stage, in which the cyanosis is usually general but is pronounced only at certain places, such as the ears, lips, and finger-tips.

The blood samples have all been drawn and kept after the procedure given in previous publications (15, 16).

The total oxygen capacity is determined either directly by the Van Slyke method (17) or (in a minority of the cases) indirectly by calculation from the color index (Haldane's method). The venous oxygen and carbon dioxide are measured by the Van Slyke method (18). The carbon dioxide is determined on whole blood kept under oil for a very short time (19).

Determinations.

The results of the estimation of the cyanosis and the determination on the blood are given in Tables I to III. The figures of the first column indicate the serial number of the determination. In the second column are given in volumes per cent the values of the carbon dioxide (in Table I), the oxygen (Table II), and the oxygen unsaturation (Table III) of the venous blood. In Column 3 are the remarks on cyanosis, which are to be compared with the blood findings in Column 2. The figures in the fourth column indicate the serial numbers of corresponding determinations in Tables I to VI of Paper II (20), in which details about the patients and the blood determinations are given. In the last column are remarks about the condition of the individual from whom the blood has been drawn.

TABLE I.

Carbon Dioxide Content of the Venous Blood (Whole Blood Kept under Oil) in a Series of Cyanotic and Non-Cyanotic Individuals.

Determination No.	Carbon dioxide of venous blood kept under oil.	Cyano-sis.	Serial No. of determination in Tables I to VI (Paper II).	Remarks (diagnosis, etc.).
	<i>vol. per cent</i>			
1	69.8	—	Not published.	Anemia (hemoglobin 44 per cent by Haldane's method).
2	67.7	—	47	Pneumonia.
3	62.1	+	4	{ Normal individual after exercise.
4	61.7	—	3	{ " " resting.
5	61.5	+++	48	Pneumonia.
6	58.2	+++	31	Normal individual; artificial stasis on arm.
7	58.1	—	9	{ Slightly decompensated heart failure; rest.
8	58.1	++	10	{ " " " exercise.
9	57.0	—	Not published.	Normal individual; rest.
10	56.6	+	23	Decompensated heart failure.
11	56.6	+	30	Normal individual; artificial stasis on arm.
12	56.5	—	11	Decompensated heart failure; rest.
13	55.9	+	27	" " " "
14	55.6	++	12	" " " exercise.
15	55.4	+	24	" " " rest.
16	55.1	—	Not published.	Normal individual; rest.
17	54.7	++	28	Decompensated heart failure; rest.
18	54.1	—	Not published.	Normal individual; rest.
19	53.8	+	30	Decompensated heart failure; rest.
20	53.0	+	51	Normal individual inhaling air with low oxygen percentage.
21	52.1	—	50	{ Normal individual; rest.
22	51.9	—	29	{ " " " "
23	51.5	+	34	Congenital heart failure; cyanosis.
24	51.3	+++	52	Normal individual inhaling air with low oxygen percentage.
25	50.8	—	49	Pneumonia.
26	50.5	—	1	{ Normal individual; rest.
27	50.5	+	2	{ " " exercise.
28	49.4	—	Not published.	Cardiac neurosis; tachycardia.
29	48.3	—	26	Compensated heart failure.
30	46.8	+++	38	Congenital " " cyanosis.
31	45.2	++	13	" " " "
32	44.6	—	Not published.	Normal individual; exercise.
33	44.1	+++	14	Congenital heart failure; cyanosis; exercise.
34	42.1	+++	39	" " " " "
35	41.5	++	35	" " " " rest.

TABLE II.

Oxygen Content of the Venous Blood in a Series of Cyanotic and Non-Cyanotic Individuals.

Determina- tion No.	Oxygen content of venous blood.	Cyano- sis.	No. of determinations in Tables I to VI (Paper II).	Remarks (diagnosis, etc.).
	vol. per cent			
1	0.00	++	12	Decompensated heart failure; exercise.
2	1.16	—	Not published.	Anemia (34 per cent hemoglobin by Haldane's method).
3	1.19	—	" "	Anemia (32 per cent hemoglobin by Haldane's method).
4	1.48	—	" "	Anemia (44 per cent hemoglobin by Haldane's method).
5	1.98	—	" "	Anemia (44 per cent hemoglobin by Haldane's method).
6	2.20	—	" "	Anemia (43 per cent hemoglobin by Haldane's method).
7	2.58	+	10	Decompensated heart failure.
8	3.14	+	20	" " "
9	3.79	+	15	" " "
10	3.95	—	Not published.	Anemia (60 per cent hemoglobin by Haldane's method).
11	4.10	+	22	Decompensated heart failure.
12	4.13	+	24	" " "
13	4.20	+	4	Normal individual; exercise.
14	4.29	+	19	Decompensated heart failure.
15	4.87	—	Not published.	Anemia (55 per cent hemoglobin by Haldane's method).
16	4.95	+++	45	Pneumonia.
17	5.09	—	Not published.	Anemia (55 per cent hemoglobin by Haldane's method).
18	5.23	+	17	Decompensated heart failure.
19	5.42	+	6	Normal individual; exercise.
20	5.72	+	2	" " "
21	6.32	?	21	Decompensated heart failure.
22	6.73	—	47	Pneumonia.
23	6.75	+++	43	"
24	7.00	+	27	Decompensated heart failure.
25	7.32	—	Not published.	Anemia (68 per cent hemoglobin by Haldane's method).
26	7.37	+++	52	Normal individual inhaling air with low oxygen percentage.
27	7.62	+	25	Decompensated heart failure.
28	7.76	+++	46	Pneumonia.

TABLE II—*Concluded.*

Determina- tion No.	Oxygen content of venous blood.	Cyano- sis.	No. of determinations in Tables I to VI (Paper II).	Remarks (diagnosis, etc.).
	<i>vol. per cent</i>			
29	8.05	—	11	Decompensated heart failure.
30	8.17	+++	31	Normal individual; artificial stasis on arm.
31	8.25	+	23	Decompensated heart failure.
32	8.52	+++	40	Pneumonia.
33	8.54	—	Not published.	Normal individual; slight exercise.
34	8.55	+	16	Decompensated heart failure.
35	8.62	+	28	“ “ “
36	8.78	?	8	Compensated “ “ exercise.
37	8.88	—	9	Decompensated “ “ rest.
38	9.09	+	30	Normal individual; artificial stasis on arm.
39	9.66	?	26	Decompensated heart failure.
40	9.80	+++	14	Congenital “ “ exercise.
41	10.13	—	Not published.	Anemia (93 per cent hemoglobin by Haldane's method).
42	11.40	++	34	Congenital heart failure.
43	11.40	+++	41	Pneumonia.
44	11.51	+++	39	Congenital heart failure.
45	12.26	—	49	Pneumonia.
46	12.62	+	51	Normal individual inhaling air with low oxygen percentage.
47	13.41	—	7	Compensated heart failure.
48	13.87	+++	38	Congenital “ “
49	14.00	—	5	Normal individual; rest.
50	14.19	—	3	“ “ “
51	15.28	—	29	“ “ “
52	15.40	+++	37	Congenital heart failure.
53	16.20	—	26	Compensated “ “
54	16.22	+++	48	Pneumonia.
55	16.31	—	1	Normal individual; rest.
56	17.03	—	50	“ “ “
57	17.44	+++	36	Congenital heart failure.
58	17.45	++	35	“ “ “
59	17.88	++	13	“ “ “
60	18.08	++	33	“ “ “
61	19.20	++	32	“ “ “
62	28.00	—	Not published.	Polycythemia (Vaquez' disease).

TABLE III.

Oxygen Unsaturation of the Venous Blood in a Series of Cyanotic and Non-Cyanotic Individuals.

Determina- tion No.	Oxygen unsatu- ration of venous blood	Cyano- sis.	No. of determinations in Tables I to VI (Paper II).	Remarks (diagnosis, etc.).
	<i>vol per cent</i>			
1	20.89	+++	39	Congenital heart failure.
2	19.82	++	12	Decompensated " " exercise.
3	18.48	+++	14	Congenital " " "
4	17.45	+++	38	" " " rest.
5	17.30	+	24	Decompensated " " "
6	17.24	+	10	" " " exercise.
7	16.62	++	34	Congenital " " "
8	15.88	+++	37	" " "
9	15.41	+	15	Decompensated " "
10	14.68	+	2	Normal individual; exercise.
11	14.60	+	4	" " "
12	14.26	+++	36	Congenital heart failure.
13	14.02	+	27	Decompensated " "
14	13.97	+	19	" " "
15	13.86	+	30	" " "
16	13.62	+	6	Normal individual; exercise.
17	13.48	+	20	Decompensated heart failure.
18	13.43	+++	52	Normal resting individual inspiring air with low percentage of oxygen.
19	12.88	—	Not published.	Normal individual; exercise.
20	12.62	++	34	Congenital heart failure.
21	12.61	+++	45	Pneumonia.
22	12.53	+++	31	Normal individual; artificial stasis on arm.
23	12.52	+	22	Decompensated heart failure.
24	12.33	+	19	" " "
25	12.16	+++	42	Pneumonia.
26	12.15	+	23	Decompensated heart failure.
27	11.58	++	28	" " "
28	10.94	—	9	" " "
29	10.92	++	33	} Congenital heart failure.
30	10.83	++	35	
31	10.81	+++	43	Pneumonia.
32	10.68	—	Not published.	Cardiac neurosis; exercise.
33	10.65	+	16	Decompensated heart failure.
34	10.47	?	8	" " " exercise.
35	10.40	++	13	Congenital " "

TABLE III—*Concluded*.

Determina- tion No.	Oxygen unsatur- ation of venous blood.	Cyano- sis.	No. of determinations in Tables I to VI (Paper II).	Remarks (diagnosis, etc.).
	<i>vol. per cent</i>			
36	10.30	?	21	Decompensated heart failure.
37	10.21	+	30	Normal individual; artificial stasis on arm.
38	9.90	++	32	Congenital heart failure.
39	9.54	?	18	Decompensated " "
40	9.40	—	Not published.	Normal individual; exercise.
41	8.86	+++	46	Pneumonia.
42	8.78	+++	48	"
43	8.68	+++	40	"
44	8.58	—	Not published.	Normal individual; exercise.
45	8.18	+	51	" " inhaling air with low oxygen percentage.
46	8.03	++	44	Pneumonia.
47	7.98	—	Not published.	Cardiac neurosis; rest.
48	7.93	++	41	Pneumonia.
49	7.12	—	Not published.	Normal individual; rest.
50	6.19	—	47	Pneumonia.
51	5.84	—	7	Compensated heart failure; rest.
52	5.20	—	5	Normal individual; rest.
53	5.01	—	3	" " "
54	4.82	—	26	" " "
55	4.72	—	29	" " "
56	3.79	—	1	" " "
57	3.77	—	50	" " "
58	2.35	—	49	" " "

In Table I are given the values in volumes per cent of the carbon dioxide of the whole (unchanged) venous blood. In twenty-three instances cyanosis of different degrees was encountered; in twelve instances no cyanosis was found. The table is arranged according to decreasing values of carbon dioxide. It is easily seen that no relation exists between the carbon dioxide content of the (venous) blood and cyanosis. A cyanotic color may be associated with low as well as with high values of carbon dioxide.²

² The upper normal limit of the carbon dioxide of the venous blood (whole blood drawn and kept under oil) is approximately 65 volumes per cent, the lower limit about 40 volumes per cent. Determinations of the carbon dioxide of whole blood in a series of individuals will be published later.

In Table II are given the values of the oxygen of the venous blood in volumes per cent. The table is arranged according to increasing values of oxygen content. A glance at the table shows that the amount of oxygen left in the venous blood³ has no simple relation to the production of cyanosis. One may find cyanotic patients with very low values for the venous oxygen and others with very high values. As in Table I, the non-cyanotic are distributed over the same range.

The table shows that one may find very high (28.00 volumes per cent), as well as extremely low values for the venous oxygen. In one case (Determination 1) the venous blood contained no oxygen. The importance of this fact is discussed in a previous paper (21).

Table III presents the values of the oxygen unsaturation⁴ arranged according to decreasing values. There is no doubt that some intimate relation exists between the oxygen unsaturation and the cyanosis. The most striking feature is undoubtedly that cyanosis is not associated with values of the oxygen unsaturation below about 8 volumes per cent (7.93 volumes per cent for No. 48).⁵ This is of considerable importance because the upper limit of oxygen unsaturation in resting normal individuals has previously (16) been found to be about 8 volumes per cent.

This does not at all mean that we find a cyanotic color in all instances in which the oxygen unsaturation is above that limit. It is seen that values of the oxygen unsaturation from 8 to about 13.0 volumes per cent (12.88 volumes per cent for No. 19) may be encountered in cyanotic as well as in non-cyanotic individuals. Values above 13.0 volumes per cent have in all instances been associated with cyanosis. The highest observed value for the oxygen unsaturation is about 21 volumes per cent (20.89).

³ In a previous publication (21) this is termed rest oxygen or reserve oxygen of the blood, analogous to the reserve force of the heart muscles.

⁴ That is, the difference between the total oxygen capacity of the blood and the venous oxygen.

⁵ In several other unpublished determinations of oxygen unsaturation the same holds true.

SUMMARY.

These data prove that abnormally high oxygen unsaturation of the blood is a cause of cyanosis.

The fact that the lowest value of oxygen unsaturation (in the venous blood) associated with cyanosis is about 8 volumes per cent seems to indicate that this amount of reduced hemoglobin is the lowest capable of producing a cyanotic color. We shall later discuss this point more in detail.

Table III shows furthermore that in spite of the fact that cyanosis is due to abnormally high oxygen unsaturation, no proportionality exists between the intensity of the blue color and the amount of reduced hemoglobin. This may in small part be due to individual peculiarities of the skin and subcutaneous tissue, which are known to influence in anemia the relation between paleness of the skin and the decrease in hemoglobin.

We shall, however, in the succeeding paper show that the main cause of the disproportionality between cyanosis and venous oxygen unsaturation is found in another factor, the recognition of which throws a clearer light on the pathogenesis of cyanosis, and explains why we may find values of oxygen unsaturation as high as 13 volumes per cent in non-cyanotic individuals.

BIBLIOGRAPHY.

1. de Senac, J. B., *Traité de la structure du cœur, de son action et des maladies*, Paris, 1749.
2. Morgagni, J. B., *De sedibus et causis morborum per anatomen indagatis libri quinque*, Venice, 1761.
3. Grancher, J., quoted from Hirschfelder, *Diseases of the heart and aorta*, Philadelphia and London, 2nd edition, 1913, 552.
4. Knapp, quoted from Posey, *Am. J. Med. Sc.*, 1905, cxxx, 415.
5. Panum, P. L., *Untersuchungen über die Entstehung der Missbildungen zunächst in den Eiern der Vögel*, Berlin, 1860.
6. Dareste, C., *Recherches sur la production artificielle des monstruosités, ou essais de téra togénie expérimentale*, Paris, 2nd edition, 1891.
7. Stockard, C. R., *J. Exp. Zool.*, 1906, iii, 99.
8. Bardeen, C. R., *J. Exp. Zool.*, 1907, iv, 1.
9. Loeb, J., *Arch. ges. Physiol.*, 1893, liv, 528.
10. Krehl, L., *Deutsch. Arch. klin. Med.*, 1889, xliv, 426.

11. Vaquez, H., *Compt. rend. Soc. biol.*, 1892, iv, series 9, 384.
12. von Bamberger, H., *Lehrbuch der Krankheiten des Herzens*, Vienna, 1857.
13. Lundsgaard, C., *J. Exp. Med.*, 1918, xxvii, 219.
14. Langstroth, L., *Arch. Int. Med.*, 1919, xxiii, 56.
15. Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxiii, 133.
16. Lundsgaard, C., *J. Exp. Med.*, 1918, xxvii, 179.
17. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
18. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
19. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 307.
20. Lundsgaard, C., *J. Exp. Med.*, 1919, xxx, 271.
21. Lundsgaard, C., *J. Exp. Med.*, 1919, xxx, 147.



STUDIES ON CYANOSIS.

II. SECONDARY CAUSES OF CYANOSIS.

By CHRISTEN LUNDSGAARD, M.D.

(From the Medical Clinic of the University of Copenhagen, Copenhagen, Denmark.)

(Received for publication, May 23, 1919.)

By secondary causes of cyanosis we mean the conditions, physiological or pathological, which can increase the oxygen unsaturation of the venous blood to a degree great enough to give cyanosis. They may be divided into two groups according to the manner in which the oxygen unsaturation of the venous blood is increased: (1) conditions which increase the reduction of the normally saturated arterial blood in the capillaries; (2) conditions which prevent complete oxidation of the venous blood in the lungs.

To decide which of the two factors is responsible in a given case may be somewhat difficult. It could be done by drawing simultaneously samples of arterial and venous blood to find the degree of oxygen saturation in both. This I have not been able to do, because up to the present arterial punctures in patients have not been considered advisable.¹ We must in the present series of cases, therefore, confine ourselves to a judgment of the circumstances under which the cyanosis occurred. Sometimes in a given case it is difficult to decide exactly in which of the two ways cyanosis is produced. In other instances where the circumstances are less complicated, it can be done with certainty. The following is a report of a series of cyanotic patients grouped according to the way in which the cyanosis was produced.

The technique is described in the preceding paper.

¹ This is discussed in a previous publication (1) in which the determinations of the oxygen in the arterial blood by Hürter (Hürter, *Deutsch. Arch. klin. Med.*, 1912, cviii, 1) are quoted. In a recently completed work Dr. W. C. Stadie of the Hospital of The Rockefeller Institute has shown that arterial puncture can be safely performed (Stadie, W. C., *J. Exp. Med.*, 1919, xxx, 215).

*Cases in Which the Abnormally High Venous Oxygen Unsaturation
Is Probably Due to an Abnormally Great Deoxidation of
the Blood in Passing through the Capillaries.*

In normal resting individuals the reduction (deoxidation) of the arterial blood, which is supposed to leave the lungs nearly saturated with oxygen, may vary from about 8 to about 2.5 volumes per cent of oxygen, the average being 5.5 volumes per cent (1). The reduction is increased by exercise (2) if the increased oxygen consumption is not compensated by a faster blood flow through the capillaries. It is also increased in some heart diseases on account of slow circulation through the capillaries (1, 3, 4, 5). In some instances the reduction may be increased to such an extent that cyanosis occurs. Cases of both kinds (exercise and heart disease) are reported in Tables I to III. Nos. 1, 3, and 5 are determinations on normal resting individuals. The oxygen unsaturation and the carbon dioxide² of the venous blood are normal. Nos. 2, 4, and 6 are similar determinations on the same individuals immediately after exercise. The carbon dioxide is unchanged. The oxygen of the venous blood (reserve oxygen) has fallen to a rather low value, and consequently the oxygen unsaturation has increased considerably (to about 14 volumes per cent). In all instances a just visible cyanosis was discovered at the finger-tips immediately after exercise.

The fourth individual (Determinations 7 and 8) was a patient with a valvular disease compensated at rest. After slight exercise the oxygen unsaturation increased from the normal value to 10.47 volumes per cent. It was impossible to decide whether or not any cyanosis was produced by exercise in this case. Determinations 9 and 11 were done on a resting patient with a slightly decompensated valvular heart disease. The oxygen unsaturation is in both instances a little above the upper normal limit (8 volumes per cent). No cyanosis is present. After heavy exercise (Determinations 10 and 12) a moderate cyanosis was produced. The oxygen unsaturation showed a considerable increase (to about 17 and 20 volumes per cent). The carbon dioxide of the venous blood was a trifle lower after exercise.

² Carbon dioxide determination not done on the third patient.

The last individual (Determinations 13 and 14) was suffering from a congenital defect of the interventricular septum and congenital cyanosis. Even at rest he was markedly cyanotic with an oxygen unsaturation of only 10.40 volumes per cent. After heavy exercise the cyanosis had grown very intense and the oxygen unsaturation had increased to 18 volumes per cent. The carbon dioxide, which is low, was slightly decreased after exercise.

There is no doubt that the high oxygen unsaturation in the venous blood of the first three (normal) individuals after exercise (Table I) was due to increased deoxidation in the capillaries, for the reason that the lungs were and had always been perfectly normal. There appears to be no reason why the arterial blood should not be fully saturated. The same is probably the case with the next two patients, whose lungs also were normal at the period of determination. In the last case (Determinations 13 and 14) the matter is more complicated. On account of the communication between the two ventricles of the heart some blood must have been passing over into the arterial system without going through the lungs. This amount may possibly be increased by exercise and take a certain part in raising the unsaturation of the venous blood from 10 volumes per cent to 18 volumes per cent. The greatest part of the increase is, however, probably due to the increased reduction of the capillary blood by exercise. As to the way the deoxidation in the capillaries is increased, there are two possibilities; increased consumption on account of the greater metabolism, or increased deoxidation on account of slower blood flow. The blood samples were drawn from the arm after stair running; it seems unlikely that this form of exercise should be able to increase the metabolism in the arms, which were quiet. It is possible that a reflectory vasoconstriction of the capillaries in the unused areas—the arms—had produced a slower flow here in order to get more blood through the capillaries of the legs. Further investigations on this problem are in progress.

The main point is that it is experimentally shown that cyanosis can be produced by increased deoxidation in the capillaries, and that when due to this cause it seems to begin when the venous oxygen unsaturation approaches a value of 13 to 14 volumes per cent.

TABLE I.
Oxygen (Oxygen Unsaturation) and Carbon Dioxide of the Venous Blood in Normal Individuals and Patients with Compensated and Decompensated Heart Diseases at Rest and after Exercise.

Determination No.	Date.	Condition.	Total oxygen capacity (a). vol. per cent	Hemoglobin (calculated). per cent	Oxygen content of venous blood (v). vol. per cent	Oxygen unsaturation (a-v). vol. per cent	Carbon dioxide of venous blood. vol. per cent	Pulse.	Respirations per min.	Remarks on cyanosis.	Conditions at the time of bleeding.	Diagnosis and clinical notes.
1	1919 Mar. 22	Rest.	20.10	108.5	16.31	3.79	50.5	90	18	No cyanosis.	Blood drawn after 10 min. rest in lying position.	Normal individual, age 25 yrs.
2		Exercise.	20.40	110	5.72	14.68	50.5	120		Slight cyanosis of fingers.	Blood drawn immediately after running up and down one flight five times in 90 seconds.	
3	Apr. 18	Rest.	19.20	104	14.19	5.01	61.7	80	14	No cyanosis.	The same as No. 1.	Normal individual.
4		Exercise.	18.80	102	4.20	14.60	62.1	120	24	Slight cyanosis of fingers.	" " " 2.	
5	" 1	Rest.	19.20	104	14.00	5.20		62	15	No cyanosis.	" " " 1.	Normal individual, age 26 yrs.
6		Exercise.	19.04	103	5.42	13.62		115	24	Slight cyanosis of fingers.	" " " 2.	

7	Apr. 4	Rest.	19.25	104	13.41	5.84	84	20	No cyanosis.	The same as No. 1.	Male, age 26 yrs.
8		Exercise.	19.25	104	8.78	10.47	134	?	Doubtful cyanosis of fingers.	Blood drawn after walking up and down one flight twice.	Compensated mitral and aortic insufficiency; auricular fibrillation.
9	Feb. 20	Rest.	19.82	107	8.88	10.94	58.1		No cyanosis of hands or face.	The same as No. 1.	
10		Exercise.	(19.82)	(107)	2.58	17.24	58.1		Marked cyanosis of hands and face.	Blood drawn after walking up and down one flight five times.	Letter carrier, male, age 56 yrs. Compensated mitral insufficiency and stenosis.
11	" 21	Rest.	19.82	107	8.05	11.77	56.5		No cyanosis of hands or face.	The same as No. 1.	
12		Exercise.	19.82	107	0.00	19.82	55.6		Marked cyanosis of hands and face.	" " " 10.	
13	Jan. 17	Rest.	28.28	153	17.88	10.40	45.2	82	Marked cyanosis of face and hands.	" " " 1.	Male, painter, age 20 yrs. Congenital cyanosis. Defect of interventricular septum.
14		Exercise.	(28.28)	(153)	9.80	18.48	44.1	108	Heavy cyanosis of face and hands.	" " " 10.	

TABLE II.
Oxygen and Carbon Dioxide of the Venous Blood in a Series of Patients with Decompensated Heart Diseases (*All at Rest*).

Determination No.	Date.	Total oxygen capacity (a). vol. per cent	Hemoglobin. per cent	Oxygen content of ven- ous blood (v). vol. per cent	Oxygen unsaturation (a-v). vol. per cent	Carbon dioxide of ven- ous blood. vol. per cent	Pulse.	Respirations per min.	Remarks on cyanosis.	Diagnosis and clinical notes.
15	Apr. 5	(19.20)	104	3.79	15.41		72* 66	34	Moderate cyanosis of hands and face.	
16	" 14	(19.20)	104	8.55	10.65		76 74	24	Moderate cyanosis of hands and face.	Housewife, age 64 yrs. Mitral insufficiency and stenosis; au- ricular fibrillation; no râles in lungs.
17	" 19	(19.20)	(104)	5.23	13.97				Moderate cyanosis of hands and face.	
18	" 26	(19.20)	(104)	9.66	9.54				Cyanosis doubtful.	
19	" 26	(16.62)	90	4.29	12.33		72 68	20	Slight cyanosis of hands and lips.	
20	May 2	(16.62)	(90)	3.14	13.48		144 108	22	Cyanosis more marked but still slight.	Housewife, age 42 yrs. Mitral insufficiency; aortic insuffi- ciency; auricular fibrillation; no râles in lungs.
21	" 5	(16.62)	(90)	6.32	10.30		92 90	22	Cyanosis doubtful.	
22	" 10	(16.62)	(90)	4.10	12.52		72 72	22	Slight cyanosis.	

23	1919 Jan. 25	20.40	(110)	8.25	12.15	56.6	$\frac{132}{96}$	28	Moderate cyanosis of face and hands.	Housewife, age 44 yrs. Mitral insufficiency and stenosis; auricular fibrillation; no râles in lungs.
24	" 27	21.43	(116)	4.13	17.30	55.4	$\frac{128}{80}$	30	Moderate cyanosis of face and hands.	
25	" 30	21.48	(116)	7.62	13.86	53.8	$\frac{102}{96}$	32	Moderate cyanosis of face and hands.	
26	Feb. 1	21.02	114	16.20	4.82	48.3	$\frac{100}{94}$		No cyanosis.	
27	" 4	(21.02)	(114)	7.00	14.02	55.9	$\frac{102}{92}$	24	Moderate cyanosis of face and hands.	
28	Apr. 15	20.20	(109)	8.62	11.58	54.7	$\frac{-}{40}$	22	Marked cyanosis of face and hands.	Mitral stenosis; auricular fibrillation; no râles in lungs.

* The upper figure is the apex pulse, the lower the radial pulse.

In Table II a report is made of fourteen determinations on four patients with valvular heart lesions combined with auricular fibrillation. In one of these determinations an oxygen unsaturation of normal value (No. 26) was found. The patient showed no cyanosis and was fully compensated at that time. In all the other instances the oxygen unsaturation was above the upper normal limit and the patients were clinically decompensated. In eleven instances a cyanosis of slight or moderate degree was encountered. In two instances (Nos. 18 and 21) it could not be determined whether or not there was cyanosis. In these two instances the oxygen unsaturation was 9.54 and 10.30 volumes per cent. During the cyanotic periods in the case covered by Determinations 15 to 18 the oxygen unsaturation was from 10.65 to 15.41 volumes per cent.

Although it seems to be almost certain that the circulation in the instances of cyanosis shown in Table II was slower than normally and that the deoxidation consequently is due to slower blood flow and not to increased metabolism, it cannot be excluded that a part of the oxygen unsaturation of the venous blood may have been due to incomplete oxidation in the lungs. In spite of the fact that the lungs in all instances were perfectly clear, it might be possible that their function had been impaired from earlier periods of stasis.

It is probably justifiable to say that the cyanosis in these cases was produced mainly, although not exclusively, by increased deoxidation on account of slow circulation. The lowest value of oxygen unsaturation at which cyanosis was found was 10 to 11 volumes per cent, somewhat lower than in the experiments with exercise, a point which will be discussed later.

In Table III three experiments on a normal person (the writer) are reported. No. 29 is a determination of the oxygen and carbon dioxide of the venous blood at rest. The values obtained are normal. Then the blood flow was stopped in the arm by stasis by means of the blood pressure apparatus. For 2 minutes the pressure was kept at 30 mm. of mercury, allowing the arterial stream to get through; then the pressure was raised to 160 mm. (the blood pressure of the individual was $\frac{125}{80}$) for 2 minutes, and a blood sample was drawn. The analysis showed that the oxygen unsaturation had increased from 4.7

TABLE III.
Oxygen and Carbon Dioxide of the Venous Blood after Stopping of the Blood Flow in the Arm of a Normal Individual (the Writer).

Determination No.	Date.	Time.	Total oxygen capacity (a).		Hemoglobin (calculated).	Oxygen content of venous blood (v).		Oxygen unsaturation (a-v).		Carbon dioxide of venous blood.	Remarks on cyanosis of arm.		Pressure in apparatus when blood sample was drawn.		Ordinary blood pressure of the subject.
			vol. per cent	per cent		vol. per cent	per cent	vol. per cent	per cent				mm.	mm.	
29	1919 Mar. 13	3.10	(20.00)	(108)	15.28	4.72	51.9	No cyanosis.	0	125	Slight cyanosis; veins swollen. Marked " "	160	0	80	Ordinary blood pressure of the subject.
30		3.14	19.30	104	9.09	10.21	56.6		160			160			
31		3.18	20.70	112	8.17	12.53	58.2		160			160			

volumes per cent to 10 volumes per cent, and a slight cyanosis was present in the whole arm. After stasis for 4 minutes more the cyanosis was very marked; the oxygen unsaturation had risen to 12.5 volumes per cent. The stasis had increased the carbon dioxide content from 51.9 volumes per cent to 58.2, both of which values lie within the normal limits.

This experiment shows the production of cyanosis by increased deoxidation of the arterial blood in the capillaries. It is striking that the cyanosis starts at a lower unsaturation (10.2 volumes per cent) than did the cyanosis in the experiments with exercise (13 to 14 volumes per cent). It is especially noteworthy that the cyanosis when caused by retarded circulation became intense at an oxygen unsaturation of 12.5 volumes per cent, which is not much higher than the value at which it was just visible when caused by exercise. This point is discussed later.

Cases in Which Cyanosis Is Exclusively or Chiefly Due to Incomplete Oxidation of the Blood in the Lungs.

Ordinarily the arterial blood is approximately saturated with oxygen. In some instances, however, the anatomical or physical conditions are changed in such a way that complete saturation does not take place. This is the case (1) when a part of the blood does not pass the lungs on account of an abnormal communication between the arterial and venous part of the vascular system, for instance a patent ductus Botalli, or a deficient septum of the ventricles of the heart; (2) when a part of the lungs is unfitted for the aeration but still allows the blood flow, for instance in pneumonia; (3) when the partial oxygen pressure in the lungs is below a certain point, for instance at high altitudes, mountain sickness; and (4) when a part of the hemoglobin is transformed into such a form that it cannot be converted into oxyhemoglobin and still gives a dark color like reduced hemoglobin, as in methemoglobinemia.³ In all these instances (the first three of

³ In methemoglobinemia, which may give an intense cyanosis, it is not quite correct to define the cyanosis as due to increased oxygen unsaturation, for the reason that a certain part of the hemoglobin can neither take up nor give off oxygen.

which are represented by experiments in this publication) the oxygen saturation of the arterial blood is incomplete and there is a non-negligible oxygen unsaturation of the arterial blood. In the capillaries this (arterial) oxygen unsaturation is further increased on account of the (normal or abnormal) deoxidation. The oxygen unsaturation in such cases is therefore a result of two factors, (1) incomplete oxidation in the lungs and (2) reduction in the capillaries.

In a series of twelve patients and one normal individual the clinical and experimental conditions have been such that although we were unable to determine the degree of arterial unsaturation we are certain that arterial unsaturation is the cause of the abnormally high venous oxygen unsaturation causing the cyanosis. The cases fall into three groups and are reported accordingly.

In Table IV a report is given of nine determinations of the blood gases in two patients, both suffering from congenital heart disease (defective ventricular septum). In both cases rather high values for the oxygen of the venous blood were found, from 11 to 19 volumes per cent, in spite of the fact that the oxygen unsaturation of the venous blood showed values which in some instances were higher than those seen in any other case, from 9.9 to 20.89 volumes per cent. The carbon dioxide of the venous blood showed values at the very lowest limit of what can be considered normal.

The cyanosis in the first case in Table IV was fairly moderate, in the last case extremely heavy. No doubt existed that the unusually high oxygen unsaturation was not due to increased deoxidation in the capillaries, because the determinations were done after half an hour's rest, and no clinical reason for slow circulation was to be detected (no stasis, no edema, diuresis normal). The cause of increased venous oxygen unsaturation must therefore lie in incomplete oxidation in the lungs⁴ caused either by some lung disease or by a congenital heart lesion. The lungs were absolutely normal, and clinically a defect of the interventricular septum was found.

In Table V a report is given of ten determinations on ten patients suffering from pneumonia (post influenza). In one case (No. 49) the oxygen unsaturation of the venous blood was very low, 2.35 volumes per cent, which is a trifle below the lower normal limit (2.5 volumes

⁴ No reason existed for considering methemoglobinemia the cause.

TABLE IV.
Oxygen and Carbon Dioxide of the Venous Blood of Two Patients Suffering from Defect of the Interventricular Septum, Congenital Cyanosis, and Polyglobulism.

Determination No.	Date.	Total oxygen capacity (a). vol. per cent.	Hemoglobin. per cent	Oxygen content of venous blood (v). vol. per cent.	Oxygen unsaturation (a-v). vol. per cent.	Carbon dioxide of blood. vol. per cent.	Pulse.	Respirations per min.	Remarks on cyanosis.	Diagnosis and clinical notes.
32	1918 Oct. 22	(29.10)	156	19.20	9.90		86	20	Moderate cyanosis, especially of face, hands, and feet.	Male, painter, age 20 yrs. Congenital heart disease (defect of the interventricular septum); congenital cyanosis; pulmonary stenosis (?). Number of red corpuscles varied between 6.2 and 7.2 millions. No stasis or edema. Superficial veins a little more marked than usual. Veins of retina dilated. Slight clubbing of fingers and toes.
33	Nov. 5	29.00	(156)	18.08	10.92		86	20		
34	" 16	(28.02)	151	11.40	16.62	51.5	84	24		
35	1919 Jan. 16	28.28	(153)	17.45	10.83	41.5	84	22	Extremely heavy cyanosis of the skin of the whole body and of the mucous membranes.	Male, age 7 yrs. Congenital heart disease (defect of the interventricular septum); congenital cyanosis. Number of red blood corpuscles varied from 7 to 10 millions. No stasis or edema. Heavy dyspnea, increasing markedly under exercise. Superficial veins of retina dilated. Veins of fingers and toes.
13	" 17	(28.28)	(153)	17.88	10.40	45.2	82	18		
36	1918 Sept. 19	31.70	(170)	17.44	14.26		100	24		
37	" 23	(31.08)	168	15.40	15.68		90	26		
38	Dec. 15	31.32	(169)	13.87	17.45	46.8	108	36		
39	1919 Feb. 2	32.40	(175)	11.51	20.89	42.1	86	34		

TABLE V.
Oxygen and Carbon Dioxide of the Venous Blood in a Series of Patients Suffering from Pneumonia (in Influenza).

Determination No.	Date.	Total oxygen capacity (a).	Hemoglobin.	Oxygen content of venous blood (v).	Oxygen unsaturation (a-v).	Carbon dioxide of venous blood.	Pulse.	Respirations per min.	Temperature.	Degree of cyanosis.	Diagnosis and clinical notes.
	1918	vol. per cent	per cent	vol. per cent	vol. per cent	vol. per cent			°C.		
40	Oct. 15	(17.20)	93	8.52	8.68		92	38		++	Male, student, age 16 yrs. Double pneumonia.
41	" 22	(19.33)	104	11.40	7.93		96	50		++	Female, age 38 yrs. Double pneumonia.
42	" 23	(15.72)	85	3.56	12.16		132	48	40.8	++	" " 23 " " "
43	" 23	(17.56)	95	6.75	10.81		120	36	40.6	++	" " 23 " " "
44	" 29	(14.79)	80	6.76	8.03		86	48	39.7	++	" " 39 " " " and aortic stenosis.
45	" 30	(17.56)	95	4.95	12.61		120	40		++	Female.
46	" 31	(16.62)	90	7.76	8.86		100	50	40.3	++	" " age 29 yrs. Double pneumonia.
47	Nov. 9	(12.92)	70	6.73	6.19	67.7	124	36	40.0	—	Age 16 yrs. Double pneumonia.
48	Dec. 1	25.00*	(135)	16.22	8.78	61.5	84	24	39.0	++	Male, porter, age 43 yrs. Double pneumonia.
49	" 4	(14.61)	80†	12.26	2.35	50.8	84	28	38.8	—	Female, housemaid, age 19 yrs. Pneumonia involving left lower lobe.

* Total oxygen capacity directly determined (Van Slyke's method).

† Total oxygen capacity calculated from hemoglobin determination (Sahlb).

In the rest the total oxygen capacity was calculated from the hemoglobin percentage determined by Haldane's method. Nos. 41 and 49 recovered; the rest died from pneumonia.

per cent). This patient was not cyanotic. In another (No. 47) the unsaturation value was within normal limits, and just above the normal average. No cyanosis was present in this patient either.

The other eight patients were cyanotic, most of them markedly so. In these cases the oxygen unsaturation of the venous blood had values from 7.93, a trifle below the upper normal limit, to 12.61 volumes per cent. As a whole, the oxygen unsaturation is abnormally high in the venous blood from the cyanotic individuals, but by far not to the same degree as was found in normal individuals after exercise, although the cyanosis in pneumonia was much more intense and generalized. In three patients, two non-cyanotic and one cyanotic, the carbon dioxide was determined. In one of these (No. 47, non-cyanotic), it was just above the upper normal limit. In the other two it was normal. The total oxygen-combining power of the blood was in one case (No. 48) very high, corresponding to a hemoglobin percentage of 135 (verified twice). In another case (No. 41) it was a little above normal. In the rest of the cases it was decreased (hemoglobin from 95 to 70 per cent).

The decision of the pathogenesis of cyanosis in these cases is somewhat difficult for several reasons. In the first place, it is possible that it might in part be due to formation of methemoglobin. From the investigations of Butterfield and Peabody (6) it is known that a discrepancy between the amount of iron in blood and its oxygen-combining power may be produced by pneumococci on account of formation of methemoglobin. No determinations of the bacteria in the blood of the present pneumonia patients were done.

If a part of the hemoglobin is transformed into methemoglobin it may or may not be included in the oxygen unsaturation values. That depends on the method used in determining the total oxygen-combining power. If this is done directly by the Van Slyke method (7) only that part of the hemoglobin which can take up and give off oxygen is included.

By the Haldane or Palmer method a mixture of carbon monoxide hemoglobin and methemoglobin is obtained which cannot be accurately matched against the standard of pure carbon monoxide hemoglobin. Neither is it possible by the Sahli method to determine accurately the total hemoglobin if a part of it is transformed into

methemoglobin.⁵ In the determinations in Table V Haldane's method was used in eight cases, seven of which had cyanosis, Van Slyke's method in one (No. 48), and Sahli's in one (No. 49) in which no cyanosis was present. In one case (No. 41) blood and urine were examined spectroscopically. No methemoglobin was detected. This means that there is a possibility that methemoglobin may have been partly responsible for the cyanosis, although it cannot be seen from the oxygen saturation values. The rather low values for the total oxygen-combining power in some of these cases might also be interpreted as caused by transformation of some part of the hemoglobin into methemoglobin, although this is entirely conjecture.

However, the main part and probably the whole of the cyanosis must be due either to increased reduction or incomplete oxidation of the blood. As to the first possibility, nothing could be detected in the circulation signs which could indicate slow circulation (no stasis, pulse powerful, the mitral stenosis found in one patient was compensated), and the patients were all resting. But the temperature was increased, which undoubtedly caused an increase in the metabolism and this means again an increased oxygen consumption in the tissues. That fever does not necessarily cause an increased oxygen unsaturation of the venous blood is seen in No. 49, where it was even subnormal in spite of a temperature of 38.8°C. This probably means that the increased activity of the heart, which was clinically very marked, had overcompensated the increased oxygen consumption, so that the venous blood was even more arterialized than normally. It is probably justifiable to ascribe the cyanosis in these cases to incomplete oxidation of the venous blood in the lungs on account of the pneumonic infiltration, which allowed the blood to pass but did not permit the air to come in contact with it. As in the patients with congenital cyanosis, the blue color is more general and begins at a lower oxygen unsaturation value than is the case in individuals when cyanosis is caused by increased reduction of hemoglobin in the capillaries.

This is also seen in an experiment in Table VI in which the influence of low oxygen pressure on the oxygen unsaturation of the venous blood is investigated. The experiment was arranged as follows: The subject

⁵ Stadie, W. C., personal communication.

TABLE VI.
Effect of Inspiration of Decreasing Amounts of Oxygen on the Skin Color and on the Oxygen and Carbon Dioxide of the Venous Blood of a Normal Individual (the Writer).

Determination No.	Date.	Time. p. m.	Total oxygen capacity (a). vol. per cent	Hemoglobin (calculated). per cent	Oxygen content of venous blood (v). vol. per cent	Oxygen unsaturated (a-v). vol. per cent	Carbon dioxide of venous blood. vol. per cent	Pulse.	Respirations per min.	Oxygen content of expiratory air. per cent	Carbon dioxide content of expiratory air. per cent	Remarks on cyanosis.
50	1919	3.28	20.80	(112)	17.03	3.77	52.1	88	17	5.68	3.69	No cyanosis.
51	Mar. 18	3.45	(20.80)	(112)	12.62	8.18	53.0	90	24	5.53	2.77	Cyanosis starting (hands, arms, and face).
52		3.48	(20.80)	(112)	7.37	13.43	51.3	90	28			Cyanosis of skin and mucous membranes very marked.

(the writer) was lying on a bed. Through a mask respiration took place from a spirometer with a capacity of about 30 liters. By means of a double valve arrangement the exposed air on its way to the spirometer passed a tower filled with soda lime, which absorbed the carbon dioxide. By thus rebreathing into the spirometer the oxygen percentage of the air decreased regularly and no increase in the carbon dioxide took place. A propeller mixed the air in the spirometer.

In several preliminary experiments the same results were obtained as in Haldane's (8). After a certain period of rebreathing the individual became cyanotic. An analysis of the air in the spirometer would then show between 8 and 5.5 per cent oxygen; the carbon dioxide about 0.5 volume per cent. The cyanosis usually started after rebreathing for about 20 minutes, and a few minutes later it would be impossible for the individual to continue on account of dizziness, throbbing in the head and extremities, palpitation, and a choking feeling.

In the experiment reported in Table VI a sample of blood was drawn from an arm vein before the spirometer breathing started. Then the respiration from the spirometer began without any change in the position of the individual on whom the experiment was performed. After 20 minutes a slight but distinct cyanosis was to be seen on the arms and cheeks. A sample of air from the mask just outside the mouth was drawn and at the same time a blood sample was taken.

The experiment was now pushed on as far as possible and three minutes after the drawing of the second sample of blood a third sample was taken together with an air sample. At that time the cyanosis was very marked and could be seen in the skin and the mucous membranes. Although it was generalized it was most conspicuous on the hands, cheeks, nose, and ears. At this time the individual was nearly fainting and felt very uneasy. The pulse rate had increased a little and the respiration had gone up from 17 to 28.

The analyses show that the cyanosis started at a venous oxygen unsaturation of 8.18 volumes per cent and was very heavy at 13.43 volumes per cent. The oxygen of the venous blood had decreased from 17.03 volumes per cent to 7.37 volumes per cent. The carbon dioxide was practically uninfluenced and showed a normal value.

The pathogenesis of the low venous oxygen unsaturation is clear. Nothing occurred which could give an increased deoxidation in the capillaries. But oxidation in the lungs must have been incomplete on account of the low oxygen percentage found in the lung air.

It is impossible to give the exact figure of the alveolar oxygen pressure because it was impossible to get a deep expiration on account of the dyspnea. The air sample, which was taken from just outside the mouth of the individual simultaneously with the drawing of the second and third blood samples, therefore shows oxygen values a little higher than the alveolar air. The oxygen percentage was, in the first sample, 5.68, in the second 5.53; the corresponding pressure was 39.7 and 38 mm. of mercury. From this we can calculate the approximate saturation of the arterial blood, using the normal average curve for the dissociation of the oxyhemoglobin. The degree of saturation could not be far from 70 per cent. Since the total oxygen-combining power is 20.80, this gives an oxygen content of the arterial blood of 15.25 volumes per cent. The difference is the oxygen unsaturation of the arterial blood, 5.55 volumes per cent. The oxygen unsaturation of the venous blood was 8.18 volumes per cent; of this 5.55 are consequently due to arterial unsaturation, whereas 2.63 volumes per cent are the result of the reduction in the capillaries on account of the oxygen consumption.

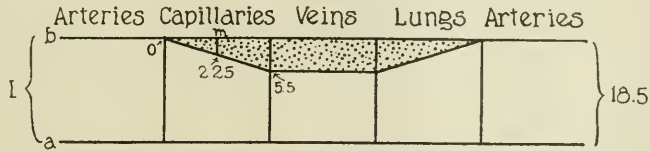
DISCUSSION.

It has been shown in this and the preceding paper (1) that an abnormally high oxygen unsaturation of the blood results in the clinical condition known as cyanosis;⁶ (2) that this increased oxygen unsaturation may be produced in two ways, (a) by an increased reduction of oxyhemoglobin to reduced hemoglobin in the peripheral capillaries, and (b) by an incomplete oxidation of the venous blood in the lungs.

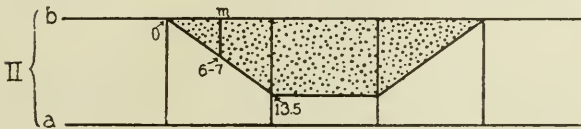
It was pointed out that no quantitative parallelism exists between the degree of cyanosis and the amount of oxygen unsaturation of the venous blood. This lack of parallelism was first thought to be

⁶ The possible production of cyanosis by methemoglobinemia has been mentioned but not discussed in detail as no data are available.

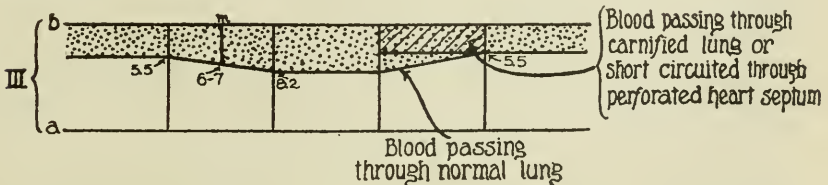
due to individual differences; for instance, of the skin and the sub-cutaneous tissue. However, the separation of the cyanotic patients into two groups according to the way the cyanosis was produced (the secondary causes) seemed to offer an opportunity for a better understanding of the problem. The results on the two groups of cyanotic patients seemed, as previously mentioned, to indicate that



Oxygen unsaturation for normal individuals.



Oxygen unsaturation when the reduction of hemoglobin in the peripheral capillaries is abnormally increased.



Oxygen unsaturation when the oxidation of hemoglobin in the lungs is incomplete.

TEXT-FIG. 1. Oxygen unsaturation of the blood in the different parts of the vascular system under various conditions. Total areas between lines *a* and *b* represent oxygen capacity, shaded areas oxygen unsaturation.

there was an important difference between them. In the first group the cyanosis was as a whole local and not very intense, beginning at a rather high degree of oxygen unsaturation of the venous blood, whereas in the second group it was usually generalized, often very intense, and it seemed to start at a low degree of oxygen unsaturation.

Text-fig. 1 represents the conditions in the different parts of the circulatory system. The distance from *a* to *b* represents the total

oxygen-combining power of the blood. The arterial system, the peripheral capillaries, the venous system, and the lungs are represented as indicated on the figure. Diagram I gives the condition in normal resting individuals. In the arteries the hemoglobin is approximately saturated with oxygen. The proportion of hemoglobin which is saturated with oxygen is represented by the white areas. In the capillaries a certain amount of oxygen is taken away by the tissues, and the highest degree of unsaturation is found at the end of the capillaries (on the average 5.5 volumes per cent). This unsaturation is unchanged until the venous blood reaches the lungs and is again oxidized.

Diagram II represents conditions in individuals, in whom the oxygen unsaturation of venous blood for some reason is increased on account of increased deoxidation in the capillaries (after exercise, slow circulation).

Diagram III gives a picture of the condition in which only part of the reduced hemoglobin of the venous blood is changed to oxyhemoglobin in the lungs. There is consequently a certain proportion of reduced hemoglobin in the arterial blood when it enters the tissue capillaries. This is further increased by the deoxidation normally occurring in the capillaries. The sum of arterial unsaturation plus reduction in the capillaries is the venous oxygen unsaturation. This condition may be found in pneumonia, some congenital heart diseases, and after respiration of air with low oxygen percentage.

All the blood samples were drawn from the veins, and the results of the analyses of these samples are compared with the cyanotic color. It is clear, however, that it is not the blood in the venous, or in the arterial system which causes the cyanosis; it is the color of the blood in the capillaries. But any part of the capillaries lying sufficiently superficially must take the same part in producing the skin color, which is to be looked upon as the result of a mixture of the condition in any small sector of the capillaries. In other words, it must be the sum of these sectors, which is responsible for the cyanosis. As to cyanosis, the sum is approximately represented by the average between the value at the beginning and the end of the capillaries. If we can calculate this average, indicated by the line *m* on the diagram, we may find the real oxygen unsaturation which is responsible

for the cyanosis. In normal individuals the values for the oxygen unsaturation at the beginning and end of passage through the capillaries are approximately 0 and 5.5 volumes per cent respectively. This indicates an average capillary oxygen unsaturation of 2.25 volumes per cent.

As an example of the condition represented in Diagram II, we shall take the results on the normal exercising individuals. The oxygen unsaturation of the arterial blood is about 0 volume per cent, that of the venous blood about 13 to 14 volumes per cent. This gives a mean capillary oxygen unsaturation of about 6.5 to 7 volumes per cent.

Diagram III may be applied to the experiment with the inspiration of air with low oxygen percentage. The arterial oxygen unsaturation was calculated to be not far from 5.55 volumes per cent. The venous oxygen unsaturation at the beginning of the cyanosis was 8.18 volumes per cent. The average is between 6 and 7 volumes per cent. In other words, the average capillary oxygen unsaturation that initiated barely visible cyanosis in selected examples from both groups, was between 6 and 7 volumes per cent, which consequently is to be looked upon as the threshold value for the cyanosis.

This view-point gives us means to a better judgment of the two factors which usually are alone available, the cyanosis and the venous oxygen unsaturation. If a cyanosis starts at a venous oxygen unsaturation lower than 13 volumes per cent, some arterial oxygen unsaturation must be present, and the more the lower the venous unsaturation is. This is often seen in heart patients, particularly in patients suffering from mitral disease, rarely in patients with aortic involvement. It is seen even if no râles are heard and no dullness is present. Almost all the results in Table II show this condition. This means that the incomplete oxidation causing arterial oxygen unsaturation plays a very important part and may be present even if no physical signs of lung involvement can be detected.

This also explains why the cyanosis in pneumonia patients starts at a very low degree of venous oxygen unsaturation and indicates that it is the lung ventilation and not the circulation which is impaired in these cases. In one patient (No. 41) the cyanosis was well developed at the venous oxygen unsaturation of 7.93 volumes per cent. This means that the arterial oxygen unsaturation was at

least between 5 and 6 volumes per cent, and from this it can be calculated that about one-third of the blood passed through lung tissue which was not ventilated.

In the experiments with artificial stasis of the arm the cyanosis begins at a venous oxygen unsaturation of 10.21 volumes per cent. This, on the basis of the above theory, would indicate the presence of some arterial unsaturation. However, it is unlikely that the arterial blood was not practically saturated with oxygen in the subject. The determination (No. 29) 4 minutes before showed that the arterial blood in this individual must have been saturated. The explanation is that back pressure from the veins caused stasis in the capillaries and reduction of an unusual proportion of the oxyhemoglobin on entering the first parts of the capillaries. Consequently the condition simulates that observed when incomplete oxygenation of the arterial blood is the cause of the presence of reduced hemoglobin at the entrance to the capillaries.

Still another phenomenon finds its explanation from the fact that it is the average capillary oxygen unsaturation which parallels the degree of cyanosis. It has been pointed out before that there must necessarily be a certain amount of hemoglobin in the blood to cause cyanosis; in other words, that patients suffering from anemia above a certain degree could not turn cyanotic. This limit can be determined by applying artificial stasis to such patients. In a series of such patients it was found that the threshold value was about 35 per cent hemoglobin, or a total oxygen-combining power of about 6.5 volumes per cent.

SUMMARY.

✓ 1. The primary cause of cyanosis is an increase in the reduced hemoglobin, or oxygen unsaturation, of the blood in the peripheral capillaries.

2. When the mean capillary oxygen unsaturation, which is calculated as the mean between venous and arterial unsaturation, and is normally about 2 to 3 volumes per cent, is increased to about 6 to 7 volumes per cent, cyanosis appears. For this reason 6 to 7 volumes per cent may be called the threshold value of mean capillary oxygen unsaturation for the incidence of cyanosis.

3. The increased mean capillary oxygen unsaturation is produced in two ways (secondary causes of cyanosis), either by an abnormally great reduction during passage through the capillaries (Text-fig. 1, Diagram II) or by a state of partial reduction in the arterial blood entering the capillaries (Text-fig. 1, Diagram III). The first condition (abnormally great reduction) occurs during exercise, or when the blood flow is retarded, as in decompensated heart condition. The second condition (partial arterial unsaturation) occurs in certain lung and heart diseases, and when the alveolar oxygen tension is greatly decreased, as at high altitudes.

4. If the blood is completely saturated with oxygen in the lungs, the oxygen unsaturation of the venous blood may increase to 13 to 14 volumes per cent before cyanosis appears.

5. If cyanosis appears at a venous oxygen unsaturation less than 13 to 14 volumes per cent, some arterial oxygen unsaturation may be assumed, and the more the lower the venous oxygen unsaturation is.

6. Even if neither râles nor dullness can be detected in the lungs, conditions may exist which prevent complete oxidation of the arterial hemoglobin. This is especially frequent in patients with mitral lesions.

7. Cyanosis cannot be produced in patients whose hemoglobin percentage is below 35 per cent on the Haldane scale (oxygen capacity of 6.5 volumes per cent).

BIBLIOGRAPHY.

1. Lundsgaard, C., *J. Exp. Med.*, 1918, xxvii, 179.
2. Lundsgaard, C., *Deutsch. Arch. klin. Med.*, 1916, cxx, 481.
3. Lundsgaard, C., *J. Exp. Med.*, 1918, xxvii, 199.
4. Lundsgaard, C., *J. Exp. Med.*, 1918, xxvii, 219.
5. Lundsgaard, C., *Deutsch. Arch. klin. Med.*, 1916, cxviii, 513.
6. Butterfield, E. E., and Peabody, F. W., *J. Exp. Med.*, 1913, xvii, 587.
7. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
8. Haldane, J. S., quoted from Starling, E. H., *Principles of human physiology*, Philadelphia, 2nd edition, 1915, 1084.

ERYTHROSIS, OR FALSE CYANOSIS.

By CHRISTEN LUNDSGAARD, M.D.

(*From the Medical Clinic of the University of Copenhagen, Copenhagen, Denmark.*)

(Received for publication, May 23, 1919.)

A general increase above the normal figure in the number of red blood corpuscles—polycythemia, erythremia—is met with under three conditions: (1) at high altitudes; (2) in certain heart diseases, particularly in congenital forms; (3) in polycythemia vera cum splenomegalia—Vaquez' disease.

Individuals with polycythemia of sufficient degree usually have an abnormal skin color. In some cases they have a reddish, in others more of a bluish color. There is in the literature some confusion as to the classification of this color. It is especially questionable whether the color in these patients is to be called cyanosis or not. The reason for the difficulty seems to be (1) the historic development of the nosology of the diseases in which polycythemia is found, and (2) the lack of a precise physiological explanation of cyanosis.

As to the first point a few historical remarks are necessary.

It is well known that a quick change from a low to a high altitude causes a series of symptoms which we call mountain sickness. The study of this disease was put on a scientific basis by Jourdanet (1) in 1864 and further developed by Paul Bert (2). The polycythemia was suggested by Paul Bert in 1882 and demonstrated by Viault (3) in 1891. Since then numerous investigators, especially Zuntz and Loewy (4) and Douglas, Haldane, Henderson, and Schneider (5) have studied this condition. Two of the main symptoms are polycythemia, and under certain circumstances blueness of the skin—cyanosis.

The polycythemia in heart diseases was first shown by Toenissen (6) in 1881 and further developed by Krehl (7), who in 1889 described a patient with polycythemia, cyanosis, and congenital heart disease with deficient septum ventriculorum. Several cases have since been described. The most frequent symptoms in congenital heart disease with deficient septum ventriculorum are polycythemia and almost constant cyanosis.

Vaquez (8) in 1892 described a patient with polycythemia who had an abnormal skin color, which he called cyanosis. He considered it a case of congenital heart failure. The postmortem examination (1893) showed no abnormalities in the heart, but an extensive enlargement of the spleen. Since then several similar cases have been reported, and this symptom complex is now recognized as a special clinical entity (Osler, 1903 (9)).

In the first description the skin color of erythrosis was called cyanosis. Chiefly for this reason, probably, most subsequent authors have described the color as cyanotic, although some of the later writers, for instance Osler and McCrae, have been inclined to describe the color not as a cyanosis, but as a congestion or ruddiness of the skin. Another reason for the confused nomenclature is probably, as already mentioned, the uncertainty in our understanding of the cause of cyanosis.

In accompanying papers (10) it is shown that the cause of cyanosis is the increase in the oxygen unsaturation of the venous blood, or rather of the blood in the capillaries. It is probably justifiable to put cyanosis in the same relation to oxygen unsaturation that anemia is to hemoglobin.

It is, therefore, of importance to determine the oxygen unsaturation in such cases. In this paper a report is made of the determination of the oxygen unsaturation in a typical case of Vaquez' disease.

The patient was a man aged 43 years. He was admitted to the hospital for duodenal ulcer. In the hospital a considerable enlargement of the spleen was found, with 181 per cent hemoglobin, 10,900,000 red blood corpuscles, 3,000 leucocytes, blood pressure 150 mm., Wassermann reaction negative. The skin and mucous membranes of the eyes, mouth, and rectum showed a deep red color.

Four determinations of the oxygen unsaturation and three of the carbon dioxide of the venous blood were done (Van Slyke's methods were used (11-13)). The results are given in Table I.

The values of the oxygen content in the venous blood are considerably greater than we usually find in normal individuals. They are increased in the same degree as the total oxygen-combining power of the blood. The oxygen unsaturation is consequently normal. The values for the carbon dioxide are low, one of them at the lowest normal limit. This may be due to the relative decrease in the serum of the

TABLE I.

Oxygen Unsaturation and Carbon Dioxide Content of the Venous Blood in a Patient with Vaquez' Disease.

Determination No.	Date.	Total oxygen capacity, directly determined (Van Slyke's method) (a).	Hemoglobin (calculated).	Oxygen content of venous blood (v).	Oxygen unsaturation (a-v).	Carbon dioxide content of whole blood.	Pulse.	Respirations per min.
		vol. per cent	per cent	vol. per cent	vol. per cent	vol. per cent		
1	1919 Jan. 8	31.50	170	26.20	5.30		70	15
2	" 13	31.50	170	26.50	5.00	58.0	66	14
3	" 21	33.40	181	28.00	5.40	56.6	68	16
4	Feb. 4	31.64	171	25.01	6.63	45.5	70	16

blood and to the abnormally high hemoglobin content of the venous blood. Neither in the skin color nor in the blood findings was any reason found for considering this man cyanotic. It was, however, evident that his color was not normal but reddish. When we consider that this particular color is one of the cardinal symptoms of the disease, it seems justifiable to give it a definite name. For this reason it is proposed to call the condition in which this color appears by the name "erythrosis." The three main symptoms of Vaquez' disease are then (1) polycythemia, (2) erythrosis of the skin and mucous membranes, and (3) splenomegaly.

SUMMARY.

1. In the venous blood of a patient with Vaquez' disease normal values were found for the oxygen unsaturation (reduced hemoglobin), although the total hemoglobin and oxygen capacity were abnormally high. The carbon dioxide content was normal.

2. The color of the skin and mucous membranes of this patient was more reddish than blue.

3. It is proposed to call the color of the skin in polycythemic patients erythrosis in order to distinguish the condition from cyanosis.

BIBLIOGRAPHY.

1. Jourdanet, D., *Le Mexique et l'Amérique tropicale; climats, hygiène et maladies*, Paris, 1864.
2. Bert, P., *Compt. rend. Acad.*, 1882, xciv, 805.
3. Viault, *Compt. rend. Acad.*, 1891, cxii, 295.
4. Zuntz, N., and Loewy, A., *Höhenklima und Bergwanderungen in ihrer Wirkung auf den Menschen*, Berlin, 1906.
5. Douglas, C. G., Haldane, E. S., Henderson, Y., and Schneider, E. C., *Phil. Tr. Roy. Soc. London*, 1913, cciii, 185.
6. Toenissen, G., *Ueber Blutkörperzählung beim gesunden und kranken Menschen*, Inaugural dissertation, Erlangen, 1881.
7. Krehl, L., *Deutsch. Arch. klin. Med.*, 1889, xlv, 426.
8. Vaquez, H., *Compt. rend. Soc. biol.*, 1892, iv, series 9, 384.
9. Osler, cited by Cabot, R. C., in Osler, W., and McCrae, T., *Modern medicine*, London, 2nd edition, 1915, iv, 679.
10. Lundsgaard, C., *J. Exp. Med.*, 1919, xxx, 259, 271.
11. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
12. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
13. Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxiii, 133.

247

SOME MORPHOLOGICAL AND BIOLOGICAL CHARACTERS OF THE SPIRILLA (VIBRIO FETUS, N. SP.) ASSOCIATED WITH DISEASE OF THE FETAL MEMBRANES IN CATTLE.

BY THEOBALD SMITH, M.D., AND MARIAN S. TAYLOR.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical
Research, Princeton, N. J.)

PLATE 16.

(Received for publication, June 3, 1919.)

In an earlier communication¹ a spirillum of definite morphological and cultural characters was described as being associated in a series of cases with what is commonly known as infectious abortion in cattle. In the following pages the statements there made concerning the biology of the spirillum are amplified and supplemented by fresh observations and by studies on the agglutinative affinities of the various strains. In all, 24 fetal strains have been kept under cultivation. Of these one (No. 308) is a slightly modified, aberrant type. All were obtained from one large herd into which cattle from outside are introduced at irregular intervals.

Morphological Characters.

In films and in hanging drop preparations from fetal fluids and cultures therefrom, the spirilla appear as fine wavy or sinuous lines of various lengths. The smallest forms appear as minute curved S-shaped lines; the longest may stretch nearly across the field of the microscope. If we assume that the spirillum is in the form of a spiral or corkscrew, the diameter of the spiral is small. The spiral is drawn out, as it were, becoming in some cultures almost a straight line. In dried and stained films the spiral becomes a shallow sinuous line (Fig. 7). No segmentations are distinguishable in the longer forms.

¹ Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

As regards size, the width of the spirillum stained in alkaline methylene blue is probably not over 0.2 to 0.3μ , the shortest form about 1.5 to 2μ long. A common size in the fetal fluids consists of about two complete turns and measures 4 to 5μ in length. As stated above, the diameter of the spiral or turn varies somewhat, but it averages about 0.5μ .

The organism stains fairly well in alkaline methylene blue provided the staining is prolonged, preferably over night. It stains much more deeply in diluted aniline water gentian violet (Fig. 6), but the former stain is to be preferred since it requires no decoloration, a process likely to decolorize the spirillum itself.

When Löffler's flagellar stain, as modified by Moore,² is applied, the organism is much thicker, as shown in Figs. 1 to 5. A flagellum is found attached to one or both poles of the spirillum. Actual counts of the spirilla of eight different strains in a given number of fields indicated that organisms having unipolar flagella are far more numerous than those with bipolar flagella. The latter may be considered relatively scarce. The unipolar forms may be individuals recently set free by division, the bipolar forms ready to divide. Longer spirilla have been seen with several short lateral flagella in addition to the terminal ones. This appearance may be due to forms which have divided but not yet separated, the flagella of the daughter cells having been formed in the meantime. The character of the flagella is best seen in the figures. They are very fine hair-like processes in large undulations or waves, of uniform width throughout.

In hanging drop preparations the shorter forms, from $\frac{1}{2}$ to 2 windings long, are very active. They shoot with lightning rapidity in straight lines across the field in all directions. Only occasionally does one reduce its velocity so that details of the movements become visible. It may then be seen in a few cases that the organism revolves around its longitudinal axis. Whether this motion is used in very rapid propulsion cannot be determined. The longer forms move sluggishly or are quiescent.

Another element brought out by staining and referred to in a former paper¹ is the granules which appear on or within the spirilla. They

² Moore, V. A., Wilder quarter century book, Ithaca, 1893, 339.

occur more commonly in old cultures and are probably degeneration forms. Usually the long spirilla contain many, but short forms are not without them. In the latter they are terminal. In the long forms they are arranged along the filament at fairly regular intervals. They stain much more deeply than the rest of the spirillum and suggest the Neisser granules of diphtheria bacilli. The feeble resistance of this spirillum to heat and drying makes any interpretation of these granules as spores improbable.

Owing to the recent extensive additions to our knowledge of spiral organisms as agents of disease and the introduction of new methods of culture, the nomenclature is not in a satisfactory state. Most writers on classification have accepted the generic designation vibrio for short, so called comma forms, and spirillum for distinctly spiral forms. The former have one polar flagellum, rarely more, the latter a tuft of polar flagella. The spiral form here described appears, as a rule, in both long spiral and short comma forms in the fetal fluids and may therefore be regarded as standing intermediate between the comma forms and true spirals. Nevertheless since this organism assumes the comma form in its early, most active stage of multiplication in cultures and has single polar flagella, the generic term vibrio appears most appropriate at the present time. It is therefore designated *Vibrio fetus*, n. sp. The term spirillum we shall continue to use in the text as a general expression for spiral organisms.

Cultural Characters.

In discussing the culture characteristics of *Vibrio fetus* we must distinguish between the earliest and later cultures. The earliest represent the still unchanged animal type, whereas later cultures may become saprophytized. The change from one to the other growth type goes on gradually and no boundary lines in terms of generations or transfers can be definitely assigned.

The earliest growths have been obtained in agar slants containing a small quantity of condensation water or added bouillon. To this a bit of tissue about $\frac{1}{8}$ gm. or an equivalent amount of stomach or other fluid or intestinal contents of the fetus is added and the tubes are sealed with sealing-wax. In such a medium the growth may be

feeble and is likely to be overlooked entirely at first. After 3 or more days of incubation two very narrow grayish white, opaque lines of growth extend upward from beneath the surface of the condensation water where the agar slant abuts against the glass. These lines, less than 1 mm. broad, are at first the only macroscopic indications of growth. Soon an exceedingly thin veil-like layer extends from each of these two lines into the capillary space between agar and glass, often meeting behind and then forming a complete barely visible layer with upper margins variably high and undulating or jagged, but rarely more than 4 cm. above the bottom of the tube. The condensation water is very feebly clouded but motile spirilla are usually found in it. Surface growth occurs only very exceptionally.

The above features persist in subsequent cultures but there are gradually added others. If defibrinated horse or other mammalian blood is added to the condensation water, a layer of growth appears on the surface of the sedimented corpuscles, which grows heavier with the number of transfers. Surface growths appear after months of cultivation either as grayish white films or as isolated, roundish, smooth, glistening colonies, 1 to 2 mm. in diameter.

With the increasing vigor of growth it becomes possible to obtain multiplication on agar slants without the blood and thereafter the strain may be maintained on plain agar in sealed tubes. In the condensation water a viscid whitish sediment made up of spirilla appears. This may be drawn out into threads. This phenomenon seems to point to a kind of mucoid degeneration which is suggested by the unstained sheaths around spirilla in Fig. 8.

In the earliest growths fluid media failed, as a rule, to induce multiplication. This failure may be due to the continually changing relation to oxygen of the different layers of fluids on account of convection currents. The agar medium provides stable conditions different at different points of the slant. The method of cultivation described differs from the methods of other workers in which deep layers of culture media, either solid, semisolid, or fluid, have been used for bacteria sensitive to oxygen. The use of slanted agar in a sealed tube gives better access to the growth than do the deep layers. It provides various degrees of oxygen tension and hence a greater variety of conditions, one of which may fit the organism to be cultivated.

With some strains a fairly vigorous multiplication has occurred in the first cultures, such as growth on the agar surface and membrane formation on the condensation water, festoons and mucoid shreds hanging down into this fluid. This is due to a specially favorable medium, which is mucus from the stomachs of the fetuses transferred to tubes when original cultures are prepared.

After thirty or more transfers with intervals up to a week multiplication has been observed in unsealed agar slants. The reduced oxygen tension in such tubes is obtained, when necessary to the organism, by growth in the capillary space between the mass of agar and the sides of the tube.

When agar was used in deep layers in sealed tubes the growth phenomena corresponded with those of *Bacillus abortus* under like conditions.

The following description applies to saprophytized strains. Fluid agar, thoroughly mixed with the spirillum, was allowed to set. Growth was gradual, appearing after incubation of 2 to 3 days as a fine white line around the circumference of the surface of the agar, and slowly growing down between the agar and the glass as a fine film. Condensation water gathered, became viscid, and growth spread over the surface of the agar. A dark line forming in the agar below the surface was observed, which at the end of a week's incubation resolved itself into a zone of individual colonies. The colonies were small, opaque, and of a yellow color. In some instances the zone appeared directly under the surface and in others it grew from 2 to 5 mm. below the surface. In unsealed tubes there was no condensation water and no growth at the surface, but the subsurface zone appeared heavier than in the sealed cultures.

In agar stabs growth developed slowly, appearing on the 2nd day along the lines of puncture. The final growth along the stab varied from 5 mm. to 3 cm. in length. If a lateral puncture was made, a fine white film gradually spread between the agar and the glass. At the points of inoculation a surface growth developed, and any slight condensation water present became viscid. In unsealed cultures no growth appeared on the surface.

Coagulated and slanted blood serum (horse) did not prove superior to the slanted agar plus a few drops of blood. No liquefaction was noted in the usually feeble growth. Growth was obtained when

defibrinated blood was incorporated with melted agar and the latter slanted. Multiplication did not take place in nutrient gelatin, either at incubator or room temperature. It is not known, therefore, whether a liquefying enzyme exists or not.

After strains had become adapted to plain slanted agar, attempts were made to induce multiplication in fluid media. Growth in simple beef peptone bouillon in sealed and unsealed tubes becomes possible, but the addition of a few drops of defibrinated blood makes multiplication more vigorous. The medium becomes faintly clouded and a slight viscid sediment is formed. A filmy stringy deposit may settle upon the sides if the tubes are slightly inclined and a viscid ring may appear at the surface, but no pellicle. Growth is not improved when paraffin oil covers the fluid column. In milk no recognizable multiplication occurs.

Biological Characters.

Since *Vibrio fetus* has many biological characters in common with *Bacillus abortus*, it would seem that these two widely separated morphological species are drawn nearer together in their physiological characters through adaptation to the same environment on the fetal membranes, causing the same sensitiveness to oxygen tension, the same fastidiousness towards culture media, and indifference to carbohydrates. Marked differences, however, do occur, such as failure on the part of *Vibrio fetus* to multiply on potato, on which *Bacillus abortus* produces a fairly rich pigmented growth.

The length of time that a sealed original culture at room temperature may contain living organisms differs from tube to tube. One culture gave rise to successful subcultures after 139 days, another failed to do so after 31 days. Subcultures on agar plus blood displayed the same differences—one dead after $2\frac{1}{2}$ weeks, another alive after 9 weeks. It is therefore important in maintaining strains to transfer once a week.

When cultures are stored in refrigerators at a temperature of 5–6°C. above freezing their vitality is reduced considerably as compared with that of cultures kept at room temperature.

The reaction of the bouillon may be carried slightly beyond the neutral point of phenolphthalein without interfering with multiplication. Indole is not formed in bouillon. Fermented bouillon with

1 per cent of dextrose, lactose, and saccharose was used in fermentation tubes to determine gas and acid production. The bulbs became uniformly clouded and a slight deposit developed; the branches remained clear. No gas was produced. After 5 days incubation the bulbs were titrated, and the reaction was found to be neutral or slightly acid. The original reaction of the bouillon had been 0.5 to

TABLE I.
Resistance to Drying.

Strain No.	Temperature.	Time of exposure.	Growth appears in.	Motility in hanging drop.
	^{°F.}		<i>days</i>	
192 (61st transfer).....	70-75	1 hr.	3	+
192 (61st ").....	70-75	2 hrs.	3	+
192 (61st ").....	70-75	3 "		-
192 (61st ").....	70-75	4 "		-
192 (61st ").....	70-75	5 "		-
192 (61st ").....	70-75	6 "		-
192 (61st ").....	70-75	1 day.		-
149 (79th ").....	70-75	1 hr.	4	+
149 (79th ").....	70-75	2 hrs.		-

TABLE II.
Resistance to Heat.

Strain No.	Temperature.	Time of exposure.	Growth appears in.	Motility in hanging drop.
	^{°C.}	<i>min.</i>	<i>days</i>	
192 (70th transfer).....	56	5		-
192 (70th ").....	56	10		-
192 (71st ").....	55	5	3	+
192 (71st ").....	55	10		-
149.....	55	5	3	+
149.....	55	10		-

0.6 per cent acid to phenolphthalein. The branch showed no acid production.

Vibrio fetus as it occurs in cultures has but little resistance to drying. Bits of linen thread impregnated with fresh active culture material resisted drying 2 hours at room temperature, but not 3 hours, as shown in Table I. Similarly resistance to heat is feeble. It is able to withstand a temperature of 55°C. for 5 minutes (Table II).

Agglutination Reactions.

The scarcity of positive differential characters of the spirilla isolated from fetuses leaves open the possibility that they may not all be alike and that many, if not all, are harmless invaders of the utero-chorionic space and the fetal digestive and respiratory tracts. To test the first possibility agglutination with immune serum was resorted to at an early stage of the work. Rabbits were treated repeatedly with doses of living spirilla injected into the abdominal cavity. After 3 to 4 weeks blood was withdrawn and the serum used in the tests shown in Tables III to VI. Before applying the agglutination test it was necessary to await the time when the various strains could be made to multiply on agar slants without blood or bits of tissue so as to eliminate any possible interference on the part of animal tissues.

At this time one of us fortunately isolated a spirillum from the spleen of a young calf which, though culturally like the fetal strains, was nevertheless wholly different in its agglutination affinities. This culture which will be taken up in another publication was used in the tests as a control. It is designated as No. 174. Somewhat later another strain was isolated from the liver of a calf 8 weeks old and the spleen of a guinea pig inoculated with duodenal contents of the same calf. This agrees serologically with *Vibrio fetus* and appears in the tables as No. 321.

The serum used is from rabbits immunized against Strains 67, 174, 256, and 317. The antigens are saline suspensions of the several strains grown on sealed agar slants at 37°C. for 4 days. The salt solution used is 0.85 per cent. The volume of fluid in each final dilution is 2 cc. The culture control contains 1 cc. of the antigen suspension and 1 cc. of salt solution, the serum control 1 cc. of a 1:10 dilution of serum and 1 cc. of normal salt solution. After a period of from 2 to 3 hours at 37°C. readings are taken. The tubes are then placed in the refrigerator over night when the final readings are made.

Tables III to V show that the agglutination reactions are of the usual kind, except for very young strains which yield a more or less paradoxical reaction, in which the clumping is feeble or absent in

TABLE III.
A. Serum from a Rabbit Treated with Cultures of *Vibrio* 67.

Vibrio No.	No. of transfer.	Dilutions of serum.										Controls.			
												Culture.	Normal serum.		
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120			1:10,240	1:20,480
67	64	C.*	C.	C.	C.	C.	C.	C.	+	+	+	+	+	—	—
149	75	"	"	"	"	"	"	"	"	"	"	"	"	—	—
159	72	"	"	"	"	"	"	"	"	"	"	"	"	Settling and clumps; cloudy.	—
174		—	—	—	—	—	—	—	—	—	—	—	—		
179	71	C.	C.	C.	C.	C.	C.	C.	+	+	+	+	+	—	—
192	?	"	"	"	"	"	"	"	"	"	"	"	"	—	—
213	56	"	"	"	"	"	"	"	"	"	"	"	"	—	—
246	25	"	"	"	"	"	"	"	"	"	"	"	"	—	—
251	23	"	"	"	"	"	"	"	"	"	"	"	"	—	—
256	21	"	"	"	"	"	"	"	"	"	"	"	"	—	—
258	19	"	"	"	"	"	"	"	"	"	"	"	"	—	—
263	14	"	"	"	"	"	"	"	"	"	"	"	"	—	—
267	17	"	"	"	"	"	"	"	"	"	"	"	"	—	—
289	8	C.	+	+	+	+	+	+	+	+	+	+	+	—	—
290	8	"	+	+	+	+	+	+	+	+	+	+	+	—	—

* In this and the following tables C. indicates complete agglutination, +++ nearly complete agglutination, ++ marked agglutination, + slight agglutination, = doubtful, and — no agglutination.

¹ when following a symbol denotes a degree of agglutination between it and the next higher symbol.

TABLE III—*Concluded.*
B. Serum from a Rabbit Treated with Cultures of Vibrio 67.

Vibrio No.	No of transfer.	Dilutions of serum.								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
67	90	C.	C.	C.	C.	C.	C.	++ ¹	-	-	-
149	97	"	"	"	"	"	"	C.	C.	-	-
318	17	"	"	++ ¹	++	++	++	+	+	-	-
159	96	"	"	C.	C.	C.	C.	C.	C.	-	-
179	89	"	"	"	"	"	++ ¹	++ ¹	++	-	-
289	25	"	"	"	"	C.	++	++	++ ¹	-	-
317	18	"	"	"	"	"	C.	C.	++ ¹	-	-
308	21	"	"	"	"	"	"	++ ¹	++	-	-
317	19	"	"	"	"	++	++	++	±	-	-
267	39	"	"	"	"	C.	C.	++ ¹	++	-	-
321	17	"	"	++ ¹	++	++	++	-	-	-	-
321	13	"	"	C.	C.	++ ¹	++	+	+	-	-
331	4	++	++	++	++	++	-	-	-	-	-
333	3	++	++	++	++	++	++	-	-	-	-
339	3	+	++	++	++	++	++	+	+	-	-

TABLE IV.
Serum of a Rabbit Treated with Cultures of Vibrio 256.

Vibrio No.	Dilutions of serum.								Controls.	
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
67	C.	C.	C.	C.	C.	+++ ¹	+++	+++	—	—
149	"	"	"	"	"	C.	+++ ¹	+++ ¹	Slight deposit; cloudy.	—
159	"	"	"	"	"	"	+++ ¹	+++ ¹	" "	—
174	—	—	—	—	—	—	—	—	—	—
179	C.	C.	C.	C.	C.	+++ ¹	+++	+++	—	—
192	"	"	"	"	"	C.	+++ ¹	+++ ¹	—	—
213	"	"	"	"	"	"	+++ ¹	+++ ¹	—	—
246	+++	"	"	"	"	"	C.	+++	—	—
251	—	—	+++	+++ ¹	+++ ¹	"	+++	+++	—	—
256	C.	C.	C.	C.	C.	"	C.	+++	—	—
258	"	"	"	"	"	"	+++ ¹	+++ ¹	—	—
263	—	+++	"	"	+++	++	+ ¹	+	—	—
267	—	—	+	++	+++	C.	+++	++	—	—
289	+	++	+++	C.	C.	+++	++	—	—	—
290	—	—	+	+++	"	C.	+++	++	—	—
321a*	+++ ¹	+++ ¹	++	+	—	—	—	—	—	—
321b†	C.	C.	C.	+++	+++	+	—	—	—	—

* Liver strain.

† Guinea pig passage strain.

TABLE V.
Serum of a Rabbit Treated with Cultures of Vibrio 317.

Vibrio No.	No. of transfer.	Dilutions of serum.								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
317	20	C.	C.	C.	C.	C.	++ ¹	+++ ¹	+++	Slight sediment.	—
67	92	+++	+++ ¹	+++ ¹	++	±	—	—	—	—	—
174	95	—	—	—	—	—	—	—	—	—	—
213	71	C.	C.	C.	C.	C.	+++	++	+	—	—
267	42	"	"	"	+++	++	+	±	±	—	—
318	21	"	"	"	C.	+++	++	+	—	—	—
290	25	"	"	"	+++	++	+	±	—	—	—

TABLE VI.
Serum of a Rabbit Treated with Cultures of Vibrio I74.

Vibrio No.	Dilutions of serum.												Controls.	
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	Culture.	Normal serum.
67	—	—	—	—	—	—	—	—	—	—	—	—	—	—
149	—	—	—	—	—	—	—	—	—	—	—	—	—	—
159	—	—	—	—	—	—	—	—	—	—	—	—	—	—
174	C.	C.	Slight clearing.	Slight clearing.	C.	C.	C.	C.	C.	C.	C.	++ ⁺	Slight deposit; cloudy.	—
179	—	—	—	—	—	—	—	—	—	—	—	—	—	—
192	—	—	—	—	—	—	—	—	—	—	—	—	Slight deposit.	—
213	—	—	—	—	—	—	—	—	—	—	—	—	—	—
246	—	—	—	—	—	—	—	—	—	—	—	—	—	—
251	—	—	—	—	—	—	—	—	—	—	—	—	—	—
256	—	—	—	—	—	—	—	—	—	—	—	—	—	—
258	—	—	—	—	—	—	—	—	—	—	—	—	—	—
263	—	—	—	—	—	—	—	—	—	—	—	—	—	—
267	—	—	—	—	—	—	—	—	—	—	—	—	—	—
289	—	—	—	—	—	—	—	—	—	—	—	—	—	—
290	—	—	—	—	—	—	—	—	—	—	—	—	—	—
308*	—	—	—	—	—	—	—	—	—	—	—	—	—	—
321a	—	—	—	—	—	—	—	—	—	—	—	—	—	—
321b	—	—	—	—	—	—	—	—	—	—	—	—	—	—
174	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	—	—	—

* This and the following tests were made at a later date.

the highest concentration and rises to a maximum to fall again. The older strains present the usual features of complete sedimentation with clarification of the supernatant fluid in the high concentrations, and gradual increase of clouding of the fluid in the lower ones. The tables indicate a close relationship between Strains 67, 256, and 317, if not identity, as the word is used in establishing a definite species of bacteria. Furthermore, the calf strain, No. 174, shows no agglutination affinities whatever with the fetal strains or with the other calf strain, No. 321.

SUMMARY.

Twenty-two fetal and two calf strains of spirilla have been studied chiefly with regard to the problem of identity. Twenty-one fetal strains are probably specifically the same. One fetal strain differs slightly from these, but in its agglutination affinities it belongs to the same group. Of two strains isolated from calves one has definite agglutination relations with the fetal strains, the other none. In the morphological and biological characters so far investigated all the strains agree closely with one another.

EXPLANATION OF PLATE 16.

Magnification, $\times 1,000$.

FIGS. 1 to 3. *Vibrio fetus* (No. 159) stained to show the polar flagella.

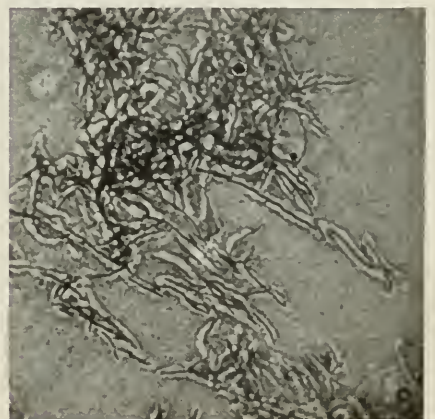
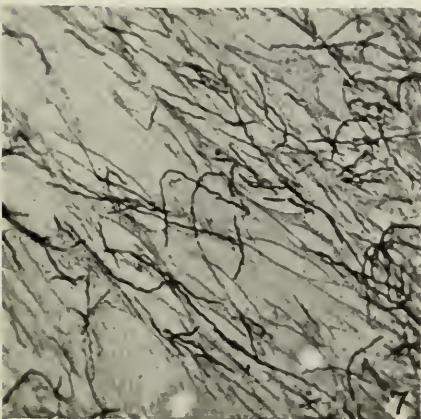
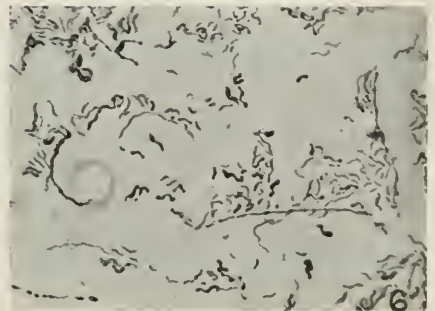
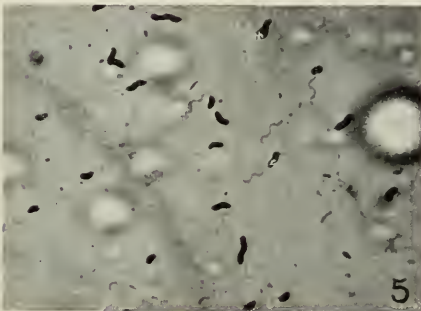
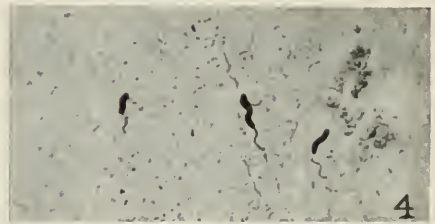
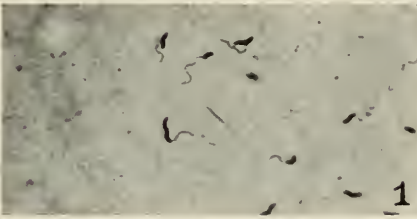
FIG. 4. *Vibrio fetus* (No. 213) stained in the same way.

FIG. 5. Spirillum from a calf (No. 174) stained in the same way.

FIG. 6. Preparation from a culture of *Vibrio fetus* (No. 290), 10th transfer, showing long and short forms, some stained deeply and with granules, others more faintly. Dilute aniline water gentian violet.

FIG. 7. Preparation from a culture of *Vibrio fetus* (No. 333) showing long forms. Stain as in Fig. 6.

FIG. 8. Another field of the same preparation showing some capsular substance, unstained, sheathing the long forms.



(Smith and Taylor: Morphology and biology of *Vibrio fetus*.)

THE ETIOLOGICAL RELATION OF SPIRILLA (VIBRIO FETUS) TO BOVINE ABORTION.*

BY THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, June 3, 1919.)

Since the summing up of the data contained in a former communication on this subject¹ investigations have been continued upon material from the same herd. From August, 1918, to June, 1919, twelve additional cases of abortion in which spirilla were isolated from fetuses in pure cultures have been studied, making twenty-six in all. In one case a slightly divergent strain was present, whose significance cannot be defined. It appears, as a rule, in long filaments, although short forms are not lacking. Thus far, motility has not been detected. The case from which this form was isolated is included in Table I as No. 308. In another paper² data on the agglutination affinities of the various strains are given and it is there shown that this aberrant strain is serologically related to the regular type. The reader is also referred to an earlier paper¹ on culture methods employed. Emphasis needs to be put on the importance of cultures from the digestive tract and the lungs. If the fetus after expulsion breathes and swallows, both tracts are likely to be contaminated with miscellaneous bacteria and spirilla will be suppressed in the cultures. Even when the fetus is dead when expelled, if the mouth and nose should lie in water or other fluids these tracts may become contaminated. Cultures from spleen, liver, and kidneys of the fetus by themselves

* This and the three accompanying papers are interrelated inasmuch as the fundamental data are the same. Duplication of statements has been avoided as far as possible and the papers should therefore be considered together in estimating the accuracy of the conclusions formulated.

¹ Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

² Smith, T., and Taylor, M. S., *J. Exp. Med.*, 1919, xxx, 299.

TABLE I.
Fetuses from Which Spirilla Were Isolated. Guinea Pig Tests for the Presence of B. abortus.

Cow No.	No. of sire.	Length of fetus. in.	Date of abortion.	No. of pregnancy after purchase.	Cultures.							Guinea pig inoculations.			
					4th stomach.	Rectum.	Lung.	Spleen.	Liver.	Kidney.	Fetal fluids or swabs.	4th stomach.	Lung.	Meconium.	Placenta or uterine discharge.
289	8, 6, 2	20	1918 Nov. 18	2	++* (Rumen ++.)	++	+	--	--	++					-
290	4, 9	24	" 19	3 (native).	+	+	++	++	++	++					-
308	?	31½	Dec. 30	1	--	+++ (Small intestine --.)	++	--	--	--					-
317	9	25	1919 Jan. 27	2	+ and other forms. ++	++	++								-
318	3, 7	26	" 27	2	++	+ and other forms.	+ and other forms.								-
331	9	8	Mar. 15	6 (native).	Two pure cultures of spirilla; one culture of paratyphoid; one culture of cocci and spirilla.										-
333	2, 10	26	" 27	1	++	++ (Colon ++.)	++ and streptococci.	--	-- and bacilli.	--					-
339	1, 2	35½	Apr. 9	3	-- (Films +.)	-- (Films of small intestine +.)	++	--	--	--					-

347	2	25 $\frac{1}{2}$	Apr. 23	4	++ (Rumen ++.) ++ and <i>B. abortus</i> .	++	++	+	--	--	--	--	--
351	7, 11	28	May 5	1		++	Miscellaneous bacteria. ++	+ and <i>B. abortus</i> .	Miscellaneous bacteria. ++	+ and <i>B. abortus</i> . ++	+	+	+
356	4, 8	26 $\frac{1}{2}$	" 23	1	++++ (One tube contains a bacillus also.) ++ (Small intestine +.)	++ (Colon +.)	++						
357	9	21 $\frac{3}{4}$	" 24	8 (native).		++ (Colon +.)	+ and contaminated.	Contaminated.	--	--	(Film +.)	--	--

* Each + stands for a pure culture of spirilla unless otherwise stated, each -- for a negative culture or negative guinea pig test with reference to *B. abortus*.

cannot be depended on to bring out the presence of spirilla in the fetus.

The notes on the pathological changes in the fetuses are omitted, since they do not differ from those of the earlier cases reported.¹ No single abnormal condition or group of conditions in the fetus could be used to prognosticate the nature of the infecting organism.

In most cases the placenta was retained. In one (No. 289) it was reported to have been discharged but it was not preserved. A portion of the placenta of No. 318 was passed attached to the fetus. It represents about one-third of the entire mass. About one-half is markedly diseased. The cotyledons are of a dirty whitish color and covered with a thin deposit which is readily washed off by repeated baths of normal salt solution. The villi after washing stand up like normal villi but lack entirely the blood-red color. Towards the more normal cotyledons some are found which contain abnormal, whitish villi and tufts or clumps of the same among the normal blood-red ones.

The intercotyledonous chorion, where cotyledons are diseased, is usually thickened and roughened. The surface is mapped out into small 1 to 2 mm. slightly raised plaques. These feel like thickened epidermis. When scraped only very little comes away. The underlying tissue is more or less edematous in localized areas, usually around and under a diseased cotyledon. Films of the cheesy covering of cotyledons stained 10 minutes and 24 hours show numerous spiral forms with from $\frac{1}{2}$ to 4 or 5 complete turns. They are all of the same fineness as regards diameter, but the amplitude of curves and the form vary somewhat. This may be due to the drying.

Sections of fixed and hardened tissue show besides a general, quite marked edema of the chorion, localized masses of nuclear debris under the epithelium, now lost. This nuclear debris results from dense collections of leucocytes, probably polynuclear, which evidently form the hard plaques of the intercotyledonous areas of the chorion, mentioned above. There is, furthermore, a general diffuse infiltration of cells into the chorion. The cotyledons contain necrotic villi without cell infiltrations.

It was stated in the preceding article¹ that *Bacillus abortus* could not be detected in the fourteen cases from which *Vibrio fetus* was iso-

lated. The same is true of those tabulated here, with the exception of one case, No. 351. In cultures from this there were found for the first time both spirilla (vibrios) and *Bacillus abortus*. The former were present in cultures from contents of the fourth stomach, colon, rectum, spleen, and kidneys. *Bacillus abortus* appeared several days later in the fourth stomach, spleen, kidney, and rectum cultures. The tubes with contents of the colon contained only spiral organisms.

The absence of *Bacillus abortus* from such a large percentage of cases is of importance. The dropping of immature calves and fetuses of various ages can no longer be referred to *Bacillus abortus* without a bacteriological study of the fetus and placenta if obtainable, or else of uterine discharges obtained with swabs and examined microscopically and by inoculation of guinea pigs. Outbreaks of abortion due solely to *Bacillus abortus* very probably occur when a herd is first attacked and therefore highly susceptible. After a time the acquired immunity modifies the conditions materially and *Bacillus abortus* becomes a relatively unimportant factor in all but first pregnancies.

Vibrio fetus may be either a true agent causing disease of the fetal membranes, or it may be simply an invader from the more external genital tract, or from the blood after the fetus has been injured or killed by other non-bacterial agencies. The simplest method to prove the truth of one or the other hypothesis is to inoculate pregnant cows. This procedure is not so easily carried out, however. In addition to the difficulties encountered in obtaining non-immune and non-infected pregnant cows is the probable attenuation of the vibrio under cultivation. This, as is described elsewhere, is subject to changes under cultivation, such as a tendency to form mucoid masses, and an adaptation to culture media without blood or bits of tissue. The quantity of growth tends to increase with successive transfers. Changes in growth characters as extensive as these most probably involve other changes, among which virulence stands first. Unfortunately, laboratory animals have so far proved refractory and cannot serve as tests of virulence. If very early transfers are inoculated into cows, the danger of introducing some hypothetical ultra-microscopic virus is much greater than in later transfers. Filtration

does not remove this possibility, since the minute spiral organisms, especially the short forms, might pass through the pores of the filter. With these difficulties in view the writer has been able to test *Vibrio fetus* upon four pregnant cows. The records of these cases are as follows:

Cow 295.—Received Nov. 21, 1918. Black Holstein of unknown age, supposed to be pregnant, according to physical examination. Reacts to tuberculin.

Dec. 10. Cow received into the left jugular vein the following suspension. Four agar slants, sealed, of vibrio (*Cow 267*, 17th transfer, inoculated Dec. 6) were used. The condensation water was first removed with pipette, then the agar moved about after adding some fresh bouillon to dislodge organisms growing between agar and glass. In all, about 10 cc. of a faintly clouded fluid were obtained. A hanging drop of this fluid shows rather long spirilla with feeble motility. Before the date of the injection the maximum range of temperature during the day was from 38.4°C. at 8 a.m. to 39.4°C. at 5.15 p.m. On the day of injection, which was made at noon, the late afternoon temperature was 40.6°C. On the following day it had fallen to normal.

On the assumption that the culture injected might have lost some of its virulence a second injection of a more recently isolated strain was made Jan. 8, 1919. Growth from sealed agar slants (*Fetus 289*, 7th transfer, 6 days old) was suspended in plain bouillon. About 9 cc. of a feebly clouded fluid were injected into a jugular vein. There was hurried respiration following the injection and some coughing. The maximum afternoon temperature was 39.8°C. Between the date of injection and the birth of a calf on Feb. 6, the morning temperature was either slightly higher than the evening temperature or else the same. The maximum morning elevation was 39.5°C., the usual temperature 39–39.3°C.

Feb. 6. After normal labor cow gave birth to a living calf. The placenta was still retained after 24 hours and the major portion of it was removed manually.³ A thick, turbid, reddish discharge with small coherent mucoid masses in it was caught in a sterile bottle by the attendant several hours after birth of calf. Fresh and stained films showed presence of cells resembling polynuclear leucocytes but with chromatin chiefly on margin and central portion nearly unstained and in most cases containing a small number of organisms, some of which were distinctly spiral, others of same diameter, but spiral form not demonstrable in them. The groups of leucocytes were in part held together by amorphous masses staining blue. Many isolated spirillar forms of one or two wave lengths present. Other bacteria scarce.

Of the placenta removed Feb. 7, the intercotyledonous areas of chorion have the minute network of vessels conspicuously injected. Small masses of a yellowish exudate, of pin-head size, sprinkled over the surface. These masses con-

³ The placenta was removed by Dr. Ernest W. Smillie and Dr. Ralph B. Little.

sist of amorphous material staining blue, remnants of cells and miscellaneous bacteria. The subchorionic tissue is not edematous. Besides these abnormal conditions, the chorion presented several areas which were thickened, opaque, whitish and slightly nodular, firm and leathery. The cotyledons vary much in appearance. None has the blood-red color of the normal mature placenta. In all there are yellowish areas, occupying more or less of the cotyledon and supplanting the normal color. This yellowish color is due largely to deposits or exudates like those described above. When a cotyledon is rinsed repeatedly in normal salt solution, the wash water becomes heavily clouded and filled with flocculi. After three or four changes these are removed and there remain in the cotyledon a variable number of yellowish villi among the dark or light red ones, which are the result of necrosis. These may form an outer zone only or occupy most of the cotyledon. In none of the many films examined were bacteria resembling *B. abortus* found. Up to the time this cow was removed for slaughter, Feb. 12, a slender mass of placental tissue about 2 feet long remained hanging from the vulva. Unfortunately, through some mistake at the abattoir, the uterus was not reserved.

In sections from the placenta there is a complete loss of chorionic epithelium. There is extensive cell infiltration with subsequent necrosis and nuclear disintegration in foci along the subepithelial layer of the chorion and in the villi. These and their branches are densely filled with nuclear debris. Occasionally a vessel is recognizable, also densely filled. The appearances indicate an active polymorphonuclear infiltration followed by necrosis. Microorganisms not definitely seen.

The calf of No. 295 appeared somewhat under weight at birth. On the 4th day it weighed 75 pounds. At birth the feet were somewhat soft and hoofs not fully developed. In all other respects the calf was normal and remained free from scours during the 6 days it was under observation.

To determine whether this cow might harbor *B. abortus* as a cause of the disease of the placenta, two guinea pigs were inoculated with uterine exudate obtained several hours after birth of calf. Both guinea pigs were normal when killed 48 days after inoculation and cultures were negative. Before the first inoculation on Nov. 28, samples of blood were withdrawn from jugular vein and the serum was tested for specific agglutinins towards *B. abortus*. Only in dilution of 1:10 was clumping observed. On Jan. 27, a second sample was withdrawn. This also was negative. Tested against *Vibrio fetus*, a very good agglutination was obtained with two older strains but not with recent strains, a condition quite regularly observed (Table II).

On Dec. 2, 1918, about 100 cc. of milk were obtained from the udder. To this were added 50 cc. of salt solution and the mixture was centrifuged at high speed for 20 minutes. Three guinea pigs were inoculated by injecting 7 cc. of the deposit and lowest strata of fluid into the peritoneal cavity of each. After 57 days the animals were killed with chloroform. There were no lesions suggestive of the presence of *B. abortus* and the cultures from the spleens remained free from growth.

The evidence is thus quite conclusive that *Bacillus abortus* was not responsible for the lesions. The microscopic examination of the early postpartum discharges showed many spirilla both free and within leucocytes. The placenta was frankly diseased and the character of the lesion, infiltration and exudation of leucocytes, corresponded with the lesions found in earlier cases, associated with spirilla. The following case is not so convincing as to the rôle of spirilla in producing disease of the chorion but the evidence is against an infection with *Bacillus abortus*.

TABLE II.
Cow 295. Agglutination Test.

Strain from Cow No.	No. of generation or transfer.	Dilutions of serum.*							
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
67	78	C.†	C.	C.	C.	C.	C.	+++	++
179	81	+++	"	"	"	"	+	—	—
267	25	—	—	+	—	—	—	—	—
289	11	—	—	—	—	—	—	—	—

* Serum from blood withdrawn Jan. 27, 1919. The tubes were kept at 37°C. for 3 hours, then in the refrigerator at about 6°C. over night.

† In this and the following table C. indicates complete clumping, and clearing of the fluid, — no change. The plus signs represent grades of clumping.

Cow 296.—White Holstein, obtained with No. 295. Assumed to be pregnant according to physical examination. Reacts to tuberculin.

Beginning Nov. 25, 1918, the temperature was taken twice daily. The highest morning temperature up to Dec. 10, the day when the animal was inoculated, was 39.2°C., the highest evening temperature 39.6°C. On Dec. 10, the growth in four sealed agar slants of spirilla from Cow 289, 4th transfer, 4 days old, was suspended in bouillon. About 10 cc. of a faintly clouded suspension were injected into the left jugular vein at noon. On this day the late afternoon temperature was 41.2°C. Next day the temperature was normal. Attendant reported violent jerky movements in abdomen of cow.

A normal calf was born early Dec. 19, 9 days after inoculation. At 9 a.m. the placenta was not completely discharged. It extended from vulva to floor, (the animal standing) where most of it lay in the bedding. A portion, including half a dozen cotyledons, which had not touched the floor was removed for examination. The intercotyledonous areas of chorion have all vessels conspicuously injected. The larger ones are cyanotic in appearance. Resting on these areas are soft, orange-colored masses of pin-head size which crush like soft cheese

between cover-slips. They consist of cell debris, fat globules, and probably leucocytes. When stained, these masses are seen to consist of polynuclear leucocytes, some epithelium, and cell debris, bound together with a cement-like substance, staining blue in alkaline methylene blue. A few spirilla-like forms detected in films of these masses after considerable search. The cotyledons in general are free from necrotic villi and the latter appear normal when covered with water. Over a number of cotyledons, however, there is the same flaky exudation, even more abundant than over the intercotyledonous areas. Films of this exudate show a few curved forms besides the cellular and amorphous masses already described. Sections of fixed and hardened portions of placenta indicate in general a still normal tissue. In slides from four different regions, however, there are a few small foci of dense polynuclear cell infiltration. One of these surrounds a thrombosed vessel in a villus.

Cultures from the exposed placenta became overgrown with a variety of bacteria. Spirilla not detected in the mixed growth. On Dec. 25, pasty, whitish, semitranslucent masses were found in bedding. These probably came from the uterus. Films showed pus cells and immense numbers of minute cocci and rod-like forms.

In order to determine whether *B. abortus* was associated with the placental lesions, three guinea pigs were inoculated with scrapings of the placenta, ground in sand and suspended in salt solution. After 43 days they were killed. There were no lesions suggesting *B. abortus* and the spleen cultures remained clear.

To determine still further the presence or absence of *B. abortus* a sample of blood was collected Nov. 28. and the serum tested against *B. abortus*. Only in a dilution of 1:10 was clumping recognizable. A second sample withdrawn Jan. 13, 1919, gave a similar result. The titer of the same serum was tested against four strains of spirilla (Table III).

On Dec. 2, 1918, about 50 cc. of milk were obtained from the udder, centrifuged, and of the lowest layer about 15 cc. were injected into the peritoneal cavity of three guinea pigs. 5 cc. into each animal. One guinea pig died in 39 days. There was extensive plastic peritonitis causing adhesion to one another of all the abdominal organs. The two remaining animals were killed with chloroform in 57 days and were found normal. Cultures from spleen remained free from growth.

The two following cases, both inoculated with strains of spirilla, were entirely negative.

Cow 334.—Received Mar. 20, 1919. Jersey grade, about 7 years old. Pregnant according to physical examination. Reacts to tuberculin.

Mar. 31, noon. Cow received into left jugular vein a suspension of spirilla isolated from Fetus 331. The cultures used were of the 3rd generation (or transfer) in sealed tubes of slanted agar containing bits of guinea pig spleen and some calf serum water. Growth after 4 days incubation feeble, but charac-

teristic. It was washed off with salt solution, bouillon added to 4 cc., and gently centrifuged to throw down minute clumps of agar, etc. The resulting suspension contained active spirilla. The temperature taken twice daily from the time of arrival fluctuated between 38° and 39°C. except on the afternoon of the day of injection, when the late afternoon temperature was 40.6°C.

An agglutination test against *B. abortus* was feebly positive at 1:20. The cow was kept under observation until Apr. 28, when she was slaughtered. The fetal membranes were normal in appearance. The fetus was 31 inches long. The only departure from the normal was the presence of a small quantity of reddish, clear fluid in the thoracic and abdominal cavities and a slight interlobular edema of the lungs. Cultures from fetal fluids (amniotic, allantoic, scrapings from chorion) and from fourth stomach of fetus were sterile after 2 weeks of incubation.

TABLE III.
Cow 296. Agglutination Test.

Strain from Cow No.	No. of genera- tion or transfer.	Dilutions of serum.*								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Serum.
67	79	C.	C.	C.	C.	+++	—	—	—	—	—
179	81	"	"	"	"	+++	—	—	—	—	—
267	24	—	—	—	—	—	—	—	—	—	—
289	10	+	+	+	—	—	—	—	—	—	—

* Serum from blood drawn Jan. 13, 1919. Tubes kept at 37°C. for 3 hours, then in refrigerator at about 6°C. over night. The control culture was suspended in salt solution. The serum control contained 5 per cent serum.

Cow 335.—Received with No. 334. Jersey grade, 9 to 10 years old. Pregnant. Reacts to tuberculin.

Mar. 31, noon. Inoculated with spirilla from Fetus 317, 13th transfer, on slanted agar. The cultures were 4 days old. Growth fair. The spirilla were removed in the same manner as for Cow 334. Active spirilla present in the final suspension. The injection was made into the left jugular vein. Within a few minutes the cow began to breathe rapidly and cough. This lasted about 2 hours. The temperature, which had been fluctuating between 38.4°C. in the morning and 39.4°C. in the late afternoon, rose to 40.6°C. on the day of injection and was normal again next day. On the same day a slight discharge from vagina was noted. A guinea pig inoculated with it showed no disease, 6 weeks later.

Agglutination tests made with *B. abortus* on Mar. 27 showed a positive clumping up to dilutions of 1:160. The cow was slaughtered with No. 334, Apr. 28, and the uterus with contents removed to the laboratory. There was no evidence of disease of the chorion. Cultures from the several fluids (allantoic, amniotic) and scrapings from chorion were negative. The fetus was 26½ inches long. Its

organs were normal with the exception of marked edema of the walls of gall bladder and the mesentery of duodenum carrying the common bile duct. Cultures of fourth stomach fluids remain sterile.

A sample of milk obtained Mar. 27 and tested on guinea pigs was later shown to be infected with *B. abortus*. This explains the agglutination titer of 1:160 towards *B. abortus* and makes it certain that this cow had passed through the abortion disease in some earlier pregnancy. It also shows that the udder may be infected with *B. abortus* and the fetal membranes and fetus remain normal. The agglutination reactions of the blood of Cows 334 and 335, taken Apr. 24, towards their own and one other strain of spirilla were quite irregular and the results are therefore withheld for further study of this test.

The foregoing experimental tests of the pathogenic rôle of *Vibrio fetus* cannot be regarded as final, but as far as they have been carried they give strong evidence that it is an etiological factor in bovine abortion. Spiral forms from four different cases were inoculated (Nos. 267, 289, 317, and 331). Only the two cases inoculated with those from Case 289 may be considered positive. Whether this negative outcome in the two remaining cases is due to decline or absence of virulence or to varying resistance on the part of the cows must be determined by additional experiments of the same kind.

SUMMARY.

The isolation in pure culture of a definite morphological entity (vibrio or spirillum) with practically the same biological characters from a series of cases of the same clinical complex (abortion) establishes a presumption in favor of the specific identity of the organisms and also in favor of the inference that such organisms are etiologically related to the diseased condition. This presumption is strengthened by the fact that disease of the fetal membranes followed the injection of pure cultures of this organism in two out of four cases.

THE BACTERIOLOGY OF BOVINE ABORTION, WITH SPECIAL REFERENCE TO ACQUIRED IMMUNITY.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, June 3, 1919.)

Since the general confirmation of Bang's discovery of a characteristic bacillus, *Bacillus abortus*, associated with abortion in cattle in different countries and the successful production of disease of the fetal membranes through infection of pregnant cows with *Bacillus abortus* in pure culture, it has been taken for granted that Bang's bacillus was the sole infectious agent, and further research into the etiology has given way to the study of methods of diagnosis, of vaccines, and their application.

The bacteriological study of cases of abortion as they occurred in a large herd in which a fair proportion of the stock was bred on the spot with accessions from other herds from time to time, continued over a period of $2\frac{1}{2}$ years, has shown that while *Bacillus abortus* may be the sole agency of abortion in certain herds, this is clearly not true for the herd under investigation.

In view of this somewhat unexpected result it seemed desirable to go over the data pertaining to the various cases in some detail. first, to see how far the bacteriological results could be depended upon to give information concerning the nature of the infectious process leading to death and expulsion of the fetus, and second, to note the bearing of the bacteriological data on the acquisition of immunity by the cow against later infection.

Methods.

The methods used for determining the presence or absence of infectious agents in the fetus and fetal membranes were those directed towards finding *Bacillus abortus* and spirilla (*Vibrio fetus*). Bits of

tissue from lungs, spleen, liver, and kidneys, and several drops of fluids from the different sections of the digestive tract of the fetus were added to slanted agar and the tubes sealed with sealing-wax. At the same time guinea pigs were inoculated with salt solution suspensions of contents of the digestive tract, portions of lungs, fetal membranes, and of swabs from the uterus whenever obtainable. For the details of the method of using guinea pigs and the results obtainable, the reader is referred to the communication by Smillie.¹

In general, cultures will not succeed unless the amounts of fluid or tissue transferred to culture tubes are adequate. Usually bits of tissue the size of small beans or fluids of equivalent amount are sufficient. But even with these the growth may be limited to the condensation water and the adjacent portion of the slant on account of the few bacteria contained in the material.

As to the relative delicacy of the two methods—cultures direct from the fetus and guinea pig inoculations—in revealing the presence of a very few abortion bacilli, it may be stated that in most cases direct cultures and guinea pig inoculations agreed in being either both positive or both negative. In a few cases, however, in which the inoculation of guinea pigs failed to produce either manifest lesions or positive cultures from the spleen, the direct cultures showed the presence of a very few abortion bacilli. The method of direct culture may therefore be considered superior to the guinea pig test under conditions assuring pure cultures.

Under other conditions the use of guinea pigs must replace largely the cultures. Fetuses over 7½ months old may be born alive. In that case they inspire a little air and swallow some saliva or bedding before they die. The invasion of miscellaneous bacteria goes on very rapidly and cultures with lung tissue or fluids from the stomachs will usually be overgrown with a variety of forms. Guinea pigs inoculated with such material may show the presence of *Bacillus abortus* unless the invasion has gone too far, when the guinea pigs may rapidly succumb to a miscellaneous infection in which pathogenic anaerobes figure largely.

To obtain pure cultures of *Bacillus abortus* from the fetal mem-

¹ Smillie, E. W., *J. Exp. Med.*, 1918, xxviii, 585.

branes, the indirect method of inoculating guinea pigs is usually the only one successful, unless the placenta is promptly discharged and, if soiled, the diseased portions are repeatedly washed in sterile water or salt solution. This procedure removes much of the infection itself in the shape of infected epithelium and phagocytic cells, but it leaves enough to make isolation of *Bacillus abortus* possible. The material is transferred to an agar slant and well distributed over the surface. A loopful from this is transferred to a second, and so on to a third or fourth and the tubes are sealed.

When the placenta remains within the uterus 24 hours or longer, the miscellaneous infection becomes so great that guinea pigs inoculated with uterine fluid or suspensions of placental tissue rarely survive a septic infection resembling that due to malignant edema or *Bacillus welchii*, unless very small doses are injected and three or four guinea pigs used. Retention of the placenta *in utero* following abortion is the rule, and therefore cultures and guinea pig inoculations of discharges are likely to fail because of the predominance of other bacteria. Even when the placenta is discharged in part it may be in a condition which precludes the successful isolation of *Bacillus abortus*.

The question then arises how far the results obtained from cultures and guinea pig tests of fetal tissues and fluids can be depended on to assure a diagnosis. In other words, is the fetus invariably infected with *Bacillus abortus* when the placenta is involved? If not, what is the percentage of abortions in which *Bacillus abortus* fails to be demonstrable in the fetal organs by the method described?

This, like other questions involving bacteriological methods, can be answered only through cumulative data. It is impossible to subject the entire fetus to bacteriological tests, and the bacteria may be so scarce or so unevenly distributed in the digestive and respiratory tracts as to escape the culture tube. The data on which this paper is based contain a small percentage of cases in which both fetus and placenta were cultured and tested on guinea pigs. This material will be fully discussed in another publication, and we can merely give results here which go to show that in perhaps not more than one out of twenty-five cases were cultures from the fetus negative and guinea pig inoculations from placental tissue or uterine discharges positive.

It may on the whole be accepted, therefore, that only a very small proportion of abortions due to *Bacillus abortus* occur before the fetus has been invaded. Even in the case of certain fetuses born alive which survived from 1 to 7 days, *Bacillus abortus* was isolated from the lungs through guinea pigs. Owing to the presence of septic organisms the intestinal contents in such cases cannot be inoculated into guinea pigs without inducing a rapidly fatal septic infection in most animals.

Grouping of Cases According to the Associated Bacteria.

In the tables the cases, for convenience, have been divided into four groups: (1) the cows raised within the herd, from which *Bacillus abortus* was isolated; (2) the cows introduced into the herd from without, from which *Bacillus abortus* was isolated; (3) cases of infection with *Vibrio fetus*; (4) cases of abortion in which cultures remained sterile and guinea pig inoculations negative, or which were associated with other agencies such as *Bacillus pyogenes*.

In Table I there are 37 cases from which *Bacillus abortus* was isolated. Of these, 26 were first pregnancies, 7 were second pregnancies, 3 were third pregnancies, and 1 was a fourth pregnancy. Of the cows aborting in the second pregnancy, 4 aborted both times and 1 had a normal first gestation. Of the 3 cows aborting in the third pregnancy, one is recorded as having aborted in the second pregnancy. The other 2 have no record of previous abortions. One aborting in the fourth pregnancy has no record of earlier abortions.

In Table II are brought together all purchased cows whose fetuses or calves or fetal membranes were found infected with *Bacillus abortus*. Some of these cows had given birth just before purchase. Some gave birth soon after purchase. In the third column are given all calvings between the time of introduction into the herd and the abortion or premature delivery of calves. If the period between acquisition and the first calving thereafter is a month or less, the next pregnancy may be considered the first in which infection with *Bacillus abortus* became possible in the new surroundings. In such cases, therefore, the second pregnancy has been designated the first in the table.

TABLE I.

Native Cows, Aborting or Giving Birth to Living Calves from Which B. abortus Was Isolated.

Case No.	Length of fetus.	No. of calving.	Remarks.
2	46 cm.	2	Aborted both times.
16	Destroyed.	1	Placenta inoculated.
17	27 in.	1	
19	Calf alive.	2	
21	25 in.	1	
22	23 "	1	
26	?	1	
30	Nearly mature.	3	2 wks. premature. No record of earlier abortions.
31	27 in.	1	
33	26 "	1	
41	Nearly mature.	1	
78	28 in.	1	
86	21½ "	1	
89	Alive.	1	
105	19½ in.	3	Aborted also in second pregnancy.
120	14 "	2	First calf normal.
134	Calf alive.	1	Placenta inoculated.
146	Fetus not found.	1	" "
150	30½ in.	1	<i>In utero.</i>
153	2 cm.	2	
164	Alive.	1	
180	33 in.	1	Fetus lived several hrs.
200	21 "	1	
203	Alive.	1	
206	28 in.	1	
210	Calf alive.	1	
215	" "	1	
224	" "	1	
227	" "	1	
229	(80 lbs.)	1	
266	31 in.	3	No record of earlier abortions.
285	28½ "	1	
298	34 "	1	
301	Alive.	2	Calf killed when 7 days old. Cow aborted in Mar , 1917. (See No. 21.)
313	29 in.	4	No record of former abortions.
316	22¼ "	2	Aborted both times.
342	25¼ "	2	" " "

This group illustrates the fact, already recognized by owners of herds, that cows introduced into an infected herd from a clean herd will abort sooner or later and in this respect act as heifers raised in the herd. They have no protecting immunity. Among the twenty-five cases of Table II are sixteen which may be considered as aborting at the first opportunity; *i.e.*, during what may be called their first pregnancy in the new herd. Seven aborted in the second pregnancy and only two in the third.

Taking together the two groups of 62 cases from which *Bacillus abortus* was isolated either directly or through guinea pigs or with both methods, we find the following classes according to the number of pregnancies: Abortion at first pregnancy, 42; of these 26 were native heifers and 16 purchased cows. Abortion at second pregnancy, 14; of these 7 were native and 7 purchased cows. Abortion, at third pregnancy, 5; 3 were native cows and 2 purchased.

The group of 26 cases from which pure cultures of spirilla have been obtained has been tabulated in other publications.^{3,3} Of these only 3 were native cows. The fetuses of these represented the third, sixth, and eighth pregnancy respectively. Among the purchased cows 6 cases were first, 9 second, 5 third, and 3 fourth pregnancies since purchase.

The fourth group of cases from which neither *Bacillus abortus* nor *Vibrio fetus* was isolated forms the most miscellaneous group and one most difficult to analyze. Of the twenty-one cases sufficiently studied through cultures, guinea pig inoculations, and the histological material to deserve a place in the table, one or two may possibly belong to the group of *Bacillus abortus*. Several may come within the group of *Vibrio fetus*; two were cases of infection with *Bacillus pyogenes* in which the preceding pregnancy had been cut short by *Bacillus abortus* infection. In eight, miscellaneous, rapidly growing bacteria appeared in the cultures. Of these several may be regarded as cases in which bacteria gained entrance immediately after expulsion, the fetus being large enough to have lived a short time after birth. One was clearly a case of asphyxiation at birth (No. 126) and one regarded by the

² Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

³ Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

TABLE II.

Purchased Cows, Aborting or Giving Birth to Living Calves from Which B. abortus Was Isolated.

Case No.	Date of purchase.	Date of calvings after purchase.	Date of abortion.	Size of fetus.	No. of calvings after purchase associated with <i>B. abortus</i> .
			1917		
20	Nov. 25, 1915		Mar. 16	Alive.	1
91	Apr. 19, 1917		June 5	30 in.	1
111	Nov. 20, 1915	Sept. 29, 1916	July 16	38 "	2
129		Oct. 2, 1915	Aug. 22	Alive.	2
		" 28, 1916			
131	July 14, 1914	July 9, 1915	" 30	28 in.	3
		Aug. 12, 1916			
			1918		
205	Dec. 14, 1916		Feb. 19	Twins; 35 and 36½ in.	1
214	Apr. 19, 1917	Apr. 24, 1917	Mar. 15	Alive.	1
222	July 25, 1916		Apr. 11	"	1
226	Apr. 3, 1916	Apr. 22, 1917	" 12	"	2
253	Aug. 17, 1917	Oct. 1, 1917	June 13	25 in.	2
270	Oct. 12, 1916	" 16, 1916	Aug. 22	29½ "	2
		Sept. 6, 1917			
273	Nov. 13, 1917	Nov. 20, 1917	" 27	16 "	1
275	Apr. 19, 1917	May 26, 1917	Sept. 2	16½ "	1
276	Nov. 20, 1915	1917	" 4	20½ "	2
278	" 10, 1917		" 19	14½ "	1
281	" 13, 1917		Oct. 4	30½ "	1
305	Aug. 17, 1917	Sept. 16, 1917	Dec. 24	21½ "	1
			1919		
309	Apr. 6, 1918		Jan. 2	(58 lbs.)	1
311	Aug. 19, 1917		" 7	(38 ")	1
325	Nov. 13, 1917		Feb. 12	36 in.	1
328	Apr. 20, 1917	Aborted Apr. 13, 1918	Mar. 9	31 "	2
329	" 18, 1918		" 11	30 "	1
343	Oct. 12, 1918		Apr. 13	14½ "	1
350	Feb. 9, 1917	Aborted in Nov., 1917, and in May, 1918.	" 30	Alive.	3
351	May 12, 1918	May 25, 1918	May 5	28 in.	1

TABLE III.

Cases of Abortion from Which neither B. abortus nor Vibrio fetus Was Isolated.

Case No.	Length of fetus.	No. of preg-nancy.	Purchased or raised.	Date of abortion.	Cultures from.							Remarks.	
					Stomach.	Small and large intestines.	Meco-nium.	Lungs.	Liver.	Spleen.	Kidneys.		Fetal mem-branes.
4	(8 mos.)	?	Raised.	1916 Apr. 1		— *		—	—				No earlier abortions.
35	16½ "	1	Purchased.	" 14 1917				—	—				
103	4 in.	3	Raised.	June 24									No earlier abortions.
122	24 "	1	Purchased.	July 31	Moulds.	Moulds.			Cocci.	—	—	2 g. p. — g. p. —	
126	(75 lbs.)	2	"	Aug. 15	g. p. — —	g. p. — —		—	—	—	—	2 g. p. —	Calf probably asphyxi- ated during birth.
143	(35 ")	1	Sept. 4, 1915. Purchased Aug., 1917.	Oct. 11	g. p. —	g. p. —		—	—	—	—	2 g. p. —	Cow said to have been fighting few days be- fore abortion.
158	12½ in.	1	Purchased Feb. 16, 1917.	Nov. 6								3 g. p. —	Calved Mar. 10, 1917. Probably a case of spirillum infection.
201	34 "	2	Purchased 1917.	1918 Feb. 12	Cultures over- grown.		— 2 g. p. —	Cultures over- grown. 2 g. p. —	—	—	—		Lungs partly inflated.
202	15½ "	4	Purchased Sept. 28, 1917.	" 13	—		— g. p. —	—	—	—	—		No record of earlier abortion.

211	23 in.	4	Raised.	Mar. 13	— g. p. —	—	—	—	—	Preceding calving 2-3 wks. premature (No. 30). <i>B. abortus</i> iso- lated at that time. Aborted Oct. 22, 1917 (No. 146).
259	20 "	2	"	July 18	g. p. —	<i>B. pyo- genes.</i>	<i>B. pyo- genes.</i>	<i>B. pyo- genes.</i>	No record of earlier abortions. Lungs not inflated.	
261	30½ "	6	Purchased Sept. 28, 1912.	" 26	— g. p. —	—	—	—	Lungs not inflated.	
262	29 "	1	Purchased Dec. 5, 1916.	" 28	Strepto- coccus. g. p. —	Colon ba- cillus. g. p. —	—	Heavy growth.	Lungs not inflated.	
265	20 "	4	Raised.	Aug. 4	— g. p. —	—	Cultures contain <i>B. coli</i> types. 2 g. p. —			Calved Feb. 27, 1916. Aborted Aug. 30, 1916. Calved Dec. 20, 1917.
277	32 "	2	"	Sept. 11	Cultures over- grown. g. p. —	Cultures over- grown.	Moulds. g. p. —	—	—	Premature calf, 7½ mos. Alive. Cow calved normally Aug. 30, 1917.
279	8 "	2	"	" 27	—	—	g. p. —	—	g. p. —	Aborted Jan. 20, 1918 (first pregnancy). Not studied.
282	Alive.	5	Purchased Aug. 21, 1912.	Oct. 8	Cultures over- grown. g. p. —	—	Cultures over- grown. g. p. —	—	—	2 wks. premature.

* — indicates negative cultures; g. p. —, negative results with reference to *B. abortus* following the inoculation of guinea pigs.

TABLE III—Concluded.

Case No.	Length of fetus.	No. of pregnancy.	Purchased or raised.	Date of abortion.	Cultures from.							Remarks.	
					Stomach.	Small and large intestines.	Mesenterium.	Lungs.	Liver.	Spleen.	Kidneys.		Fetal membranes.
291	32 in.	4	Raised.	1918 Nov. 19	<i>B. pyogenes</i> . g. p.—		— g. p.—	<i>B. pyogenes</i> . g. p.—	<i>B. pyogenes</i> .			g. p.—	No record of previous abortions. Fetus dead some time before expulsion. Lungs not inflated.
300	35 "	6	Purchased Oct. 10, 1911.	Dec. 7	— g. p.—	Stomach—	— g. p.—	— g. p.—	—	—	Cultures overgrown.		
327	36 "	1	Purchased Aug. 10, 1917.	1919 Feb. 18	Cultures overgrown. g. p.—		— g. p.—	— One culture overgrown. g. p.—	—	—	—	g. p.—	
344	36½ "	3	Purchased Aug. 20, 1916.	Apr. 12	— g. p.—	Stomach—	—	— g. p.—	—	—	Contaminated.	g. p.—	Calved in 1916 and 1917.

attendants as traceable to fighting several days before expulsion of fetus (No. 143). Seven may be considered bacteria-free or sterile. How many of these are due to injury, to toxic substances, and to food factors cannot be stated. The importance of these cases, because not associated with *Bacillus abortus*, has warranted a rather detailed publication of the data in Table III.

In this group too there are no first pregnancies. Those marked first in the table refer to purchased cows and are at least second pregnancies. Thirteen are purchased and eight native cows. The pregnancies during which the fetuses were dropped prematurely range from first to sixth.

Summing up the bacteriological data from another point of view we have 47 cases from which *Bacillus abortus* was not isolated. Among the latter were 25 cases associated with *Vibrio fetus* and 1 case containing a related but not identical spirillum; 2 with *Bacillus pyogenes*; 7 with sterile digestive and respiratory tracts; and 7 with only cultures of the digestive tract and lungs fertile, due chiefly to aspiration and swallowing of miscellaneous bacteria at birth.

Acquired Immunity to Bacillus abortus.

A statement frequently made in veterinary literature and by stock owners is that cows gradually lose the tendency to abort. This tendency is in harmony with the nature of the abortion disease,—an invasion of the fetal membranes, more particularly the chorion, by infectious agents, followed by multiplication of the latter and more or less injury to the tissues attacked.

The rapidity with which immunity is acquired varies considerably. Holth⁴ quotes one observer as giving the experience of thirty veterinarians to the effect that cows tend to abort two or three times in succession. He quotes another observer as claiming that 68 per cent of aborting cows aborted but once, and Bang as stating that of 83 aborting heifers only 20 aborted next time. In most infectious diseases the young are attacked in largest numbers. Hence we would expect the largest number of abortions among primiparæ (heifers). But even here observations differ. Holth quotes an observer as

⁴ Holth, H., *Z. Infektionskrankh. Haustiere*, 1911, x, 342.

stating that only 36 per cent of the cows under his care calved normally, whereas 45 per cent of the heifers did so.

In the tables it is evident that abortion is essentially a disease of young stock and that second and third abortions are less common. If we control the data by the bacteriological results obtained, we find that relatively few cows are subject to disease of the fetal membranes due to *Bacillus abortus* twice. Abortions do occur in succession but they are due chiefly to causes other than *Bacillus abortus*. In illustration of this fact the following consecutive abortions studied bacteriologically are noteworthy. The individual abortions have been given case numbers; each pair of numbers therefore refers to one and the same cow.

Case 3.—Cow aborted Feb. 23, 1916. Had six or seven normal calves before. Fetus contained within membranes, about 6 to 7 inches long. Fetus has undergone autolytic changes. Only head, limbs, and ribs recognizable. The entire mass has a reddish, translucent, gelatinous appearance. Guinea pigs inoculated with suspensions of ground cotyledons remained well. Cultures from spleens negative. This case cannot be considered as having been examined thoroughly bacteriologically, but *B. abortus* was most probably absent.

Case 159.—The same cow aborted Nov. 7, 1917, after having calved normally Feb. 12, 1917. Fetus 16½ inches long. Some of the placenta discharged. Fetus had been dead *in utero*, probably for some time. From this case spirilla were isolated. Tests for *B. abortus* negative.

Case 146.—Holstein heifer aborted on pasture Oct. 22, 1917. Fetus could not be found. A portion of placenta removed next day. All of twelve guinea pigs inoculated from this for another experiment were found infected with *B. abortus* when chloroformed. *B. abortus* also identified in sections of placenta.

Case 259.—The same cow aborted July 18, 1918. Fetus 20 inches long. General subcutaneous, blood-tinted edema. Some of abdominal organs eaten out by some animal. Thorax full of blood-tinted fluid. Fetus probably dead some time as autolytic changes were prominent. Cultures from intact organs—lungs, liver, and kidneys—show large numbers of colonies of a bacillus diagnosed as *B. pyogenes*. Guinea pig tests of lung and meconium negative.

Case 30.—Calved Apr. 3, 1917, but calf lived 2 hours only. Probably 2 weeks premature. This cow had calved normally in 1914 and 1916. *B. abortus* was isolated from the uninflated lungs both in cultures and through guinea pigs.

Case 211.—The same cow aborted Mar. 13, 1918. Placenta retained. Fetus 23 inches long; has undergone more or less maceration *in utero*. Cuticle peels off readily. Large serous cavities filled with blood-tinted fluid. Edema of subcutis and muscular tissue, blood-stained. Cultures from this case remained sterile. Guinea pig tests also negative.

Case 19.—Cow gave birth to twin calves Mar. 13, 1917 (second pregnancy). Both very weak, unable to stand and too weak to drink. One calf died Mar. 15, of scours. Extensive fatty degeneration of liver. Many collapsed lobules throughout left lung and in ventral lobe of right lung. Two guinea pigs inoculated with suspension of ground lung tissue in salt solution became infected with *B. abortus*.

Case 290.—The same cow on Nov. 19, 1918, gave birth to a fetus 24 inches long. Placenta retained. Guinea pig inoculations negative as to *B. abortus*. Spirilla in pure culture isolated from fourth stomach and colon contents, from liver, spleen, kidney, and lungs.

Case 21.—Heifer aborted Mar. 20, 1917. Fetus about 25 inches long. General subcutaneous edema. Large serous cavities filled with blood-tinted fluid. From the placenta *B. abortus* was isolated through a guinea pig.

Case 301.—The same heifer was bred four times and gave birth on Dec. 11, 1918, to a calf apparently normal. Placenta retained; cow was discharging from vagina. A swab containing such discharge washed out in salt solution and the latter injected into two guinea pigs. From both *B. abortus* was subsequently isolated. The calf did not do well and was killed when 7 days old. One umbilical artery close to umbilicus was involved in suppuration. Multiple foci of collapse in both ventral lobes. From a guinea pig inoculated therewith *B. abortus* was isolated.

Case 246.—Fetus 7 to 8 inches long found enclosed in membranes on the morning of May 28, 1918. Autolytic changes far advanced. No odor of bacterial decomposition. Spirilla were isolated in pure culture from the lungs and liver. No other bacteria found in the fetus. Guinea pigs inoculated with material from the same organs were normal when chloroformed after 6 weeks. This cow was purchased Sept. 2, 1917, and aborted in Nov. of the same year. The fetus from this abortion was not received for study.

Case 350.—The same cow aborted a third time Apr. 30, 1919. Fetus, due June 4, alive at birth and able to stand. Weighs 57 lbs. Killed about 4 hours after birth. Autopsy shows that no food had been taken but the rumen contained besides the fetal fluid much froth from swallowed saliva. No abnormalities were detected. The placenta was retained and films from a swab inserted into uterus showed groups of bacteria resembling *B. abortus*. Cultures from the stomachs were impure mixtures, as might have been expected, but those from colon and rectum contained only *B. abortus*. A guinea pig inoculated with salt solution suspension from the swab, chloroformed after 7 weeks, was found with the lesions of *B. abortus*.

These six pairs of consecutive abortions indicate in general an acquired immunity to *Bacillus abortus*. In three pairs the first abortion was associated with *Bacillus abortus*, the second with negative cul-

tures, *Bacillus pyogenes*, and spirilla respectively. In one pair both abortions were associated with *Bacillus abortus*. In one pair *Bacillus abortus* was not detected either time. The first abortion may or may not have been associated with spirilla, since the condition of the material (advanced autolysis) did not warrant cultures, except from the spleen. These were negative. Later studies indicated that the spleen is rarely invaded by spirilla. The last case is exceptional. Of the first abortion there is no record. It may or may not have been due to *Bacillus abortus*. The second abortion was associated with spirilla. The third, associated with *Bacillus abortus*, may have been due to reinfection from the udder following loss of immunity acquired on account of the first abortion.

Were it not for agents other than *Bacillus abortus*, such as *Vibrio fetus*, and to a far less degree to miscellaneous septic and pyogenic organisms, and to unknown, non-bacterial agencies, second and later abortions by the same cow would be relatively rare. It therefore becomes necessary in the future to distinguish between the tendency of any given cow to abort repeatedly and the relation of *Bacillus abortus* to such repeated abortions. If abortions are due to a variety of infectious and non-infectious agencies, a better knowledge of abortion can only be reached by a patient, thorough study of series of individual cases, followed by the necessary experimental tests in order that these agencies may be assigned their proper place. A further analysis of this material will be found elsewhere.⁵ It is there shown that the udders of a relatively high percentage of cows become infected with *Bacillus abortus* probably during the first abortion disease. Cows while carrying *Bacillus abortus* in the udder may give birth to normal calves, or to fetuses infected with spirilla, or to sterile fetuses. Probably the udder becomes a protecting reservoir of immune bodies towards *Bacillus abortus*.

SUMMARY.

In a large herd of dairy cattle and young stock the bacteriological examination of 109 cases of abortion which included a relatively thorough study of the fetus and a study of the membranes, or swabs from

⁵ Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx, 341.

the uterus whenever obtainable, gave the following results. 62, or 57 per cent, were associated with *Bacillus abortus*. 26, or 23.8 per cent, were associated with spirilla. 2, or 1.8 per cent, were associated with *Bacillus pyogenes*. 19, or 17.4 per cent, were either sterile or else the digestive and respiratory tracts had been invaded during or after birth with miscellaneous bacteria. *Bacillus abortus* was absent according to cultures and animal tests.

Such a relatively large proportion of cases of abortion without *Bacillus abortus* as the inciting agent is noteworthy. In general *Bacillus abortus* was associated with first pregnancies. Its presence diminished rapidly in frequency in later pregnancies. Assuming in a general way that purchased cows coming from small herds were free from any immunity and that their first pregnancy in the new herd is equivalent to that of a native heifer and may be counted as the first, we have *Bacillus abortus* associated with the first pregnancy in 42, with the second in 14, with the third in 5, and with the fourth in 1. Spirilla were distributed as follows: (a) in purchased cows, first pregnancy, 6; second pregnancy, 9; third pregnancy, 5; and fourth pregnancy, 3; (b) in native cows, first pregnancy, 0; third pregnancy, 1; sixth pregnancy, 1; and eighth pregnancy, 1. The relation of infection with spirilla to acquired immunity is not clear and more data from large herds are needed to define both etiological and immunological bearings of the spirilla.

Thus far spirilla have not been encountered in native heifers of the herd giving birth the first time. A tentative explanation to be offered is that the young stock is kept segregated from the older and purchased cows until shortly before calving. The occasional discharge of a fetus among the young stock in pasture tends to keep up the disease due to *Bacillus abortus*. Later on association with older cows brings about infection with spirilla (*Vibrio fetus*) and more rarely with other possible agencies of fetal disease. On the other hand, abortions may occur among the pastured stock from time to time and remain unrecognized. Not until both groups of animals are subjected to the same daily scrutiny will it be possible to affirm that abortion associated with spirilla does or does not occur among young stock.

AN INTERPRETATION OF THE AGGLUTINATION REACTION TO *BACILLUS ABORTUS* IN 75 CASES OF BOVINE ABORTION BACTERIOLOGICALLY CONTROLLED.

By ERNEST W. SMILLIE, D.V.M., RALPH B. LITTLE, V.M.D., AND LAURA FLORENCE.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, June 11, 1919.)

In the following pages are given the results of a study of the agglutination test in 75 cases of abortion of which bacteriological data were at hand. Concerning these data and the methods employed in obtaining the results here used to interpret our findings, the reader is referred to Dr. Smith's paper.¹ Although bacteriological data on a much larger number of cases were available, yet in many instances the cows were removed from the herd before samples of blood could be taken. While this work was in progress certain discrepancies came to the surface and the search for a focus of infection with *Bacillus abortus* in the udder was deemed essential by Dr. Smith. Accordingly the milk from certain selected cases was brought into the investigation.

The existing literature on the agglutination test is voluminous and the work in most cases of high quality, yet there is lacking the bacteriological basis of a series of spontaneous cases for a more accurate interpretation of the test. The fact that Smith¹ found in 109 cases of abortion or premature expulsion of a living calf 46 not associated with *B. abortus* shows that our information in regard to the significance of the agglutination test has been built on somewhat insecure foundations.

Most writers have confined their attention to a comparison between the agglutination and the complement fixation test. This was done by Mohler and

¹ Smith, T., *J. Exp. Med.*, 1919, xxx, 325.

Traum,² Moore and Fitch,³ Larson,⁴ Rettger and White,⁵ Wall,⁶ and Zwick and Zeller.⁷ Others have contented themselves with associating the serological reactions with abortion pure and simple. Wall,⁶ Grinsted,⁸ and Moore and Fitch³ studied among other things the rise and decline of the agglutination curve. Since none of these researches bears directly upon the substance of this communication any detailed analysis would be out of place.

Methods.

Agglutination Technique.—The obtaining of blood samples from cattle is frequently difficult, since it depends on the way the animals are stabled and their degree of wildness or domestication. Those from which samples were collected for the present investigation were in the main milking cows. They were secured in stanchions, facing on a wide alleyway. A rope halter was placed on the head of the animal and the neck and head were extended by fastening the rope, so attached, to an iron rail supporting stanchions on the other side of the alley, and tightening the rope gradually until the jugular furrows were fully exposed. Strong bull nose forceps were then put on the anterior part of the nasal septum to hold the attention of the animal, and also to steady the head. One assistant can handle both the rope and bull nose forceps.

When the animal was well under control the field of operation was thoroughly wiped off with 95 per cent alcohol. Pressure was applied over the jugular vein at the posterior end of the furrow to make the vein prominent and a $2\frac{1}{2}$ inch needle No. 14 was inserted. 25 to 30 cc. of blood were collected in a sterile 50 cc. centrifuge tube. A separate, sterile needle was used for each sample. The samples were stored at room temperature until the clot had formed, after which it was separated from the sides of the tube with a heavy sterile wire.

² Mohler, J. R., and Traum, J., *U. S. Dept. Agric., Bureau of Animal Industry, Bull.* 216, 1913, 147.

³ Moore, V. A., and Fitch, C. P., *Rep. N. Y. State Vet. College*, 1912-13, 82.

⁴ Larson, W. P., *J. Infect. Dis.*, 1912, x, 178.

⁵ Rettger, L. F., and White, G. C., *Storrs Agric. Exp. Station, Bull.* 93, 1918.

⁶ Wall, S., *Z. Infektionskrankh. Haustiere*, 1911, x, 23, 132.

⁷ Zwick and Zeller, *Arb. k. Gsndtsamte.*, 1912, xliii, 1.

⁸ Grinsted, P., *Maanedsskrift f. Dyrlaeger*, 1909, xxi, Review in *Berl. tierärztl. Woch.*, 1909, xxv, 831.

The blood samples were then placed in the refrigerator for 12 hours to allow the clot to contract completely. Each tube was centrifuged for 10 minutes, to throw the clot and free corpuscles to the bottom. With sterile bulb pipettes the clear serum was transferred from the centrifuge tubes to sterile, corked test-tubes for storage. No disinfectant was added to the serum as it was found unnecessary if each step of the procedure was carried out carefully.

The antigen used was always prepared fresh as needed. A stock culture of *Bacillus abortus* recovered early in this work by Dr. Smith was used. A 48 hour slant agar culture was washed off with 2 cc. of normal saline solution and the suspension transferred to an agar surface within a rectangular 16 ounce bottle. The bottle was sealed and incubated for 72 hours at 37°C., then washed off with 20 cc. of normal saline solution, and the suspension transferred to a sterile bottle. This very dense suspension was standardized by reducing the density with normal saline solution to a point at which the translucency equals that of a 24 hour bouillon culture of the typhoid bacillus. It was then ready for use.

In order to simplify the technique as much as possible, reduce the amount of glassware used, and safeguard the operations by using the same amounts of fluids in the series of tubes in which clumping was to be observed the following method was used throughout. Eight stock dilutions of serum were used as a starting-point. To the first tube 4 cc. of salt solution and 1 cc. of undiluted serum were added to make a stock dilution of 1:5. Into each of the other tubes $2\frac{1}{2}$ cc. of salt solution were placed. Then $2\frac{1}{2}$ cc. of the 1:5 serum dilution were added to the second tube, the mixture was drawn up several times, and $2\frac{1}{2}$ cc. of this were transferred to a third tube, and so on successively to the last. Each tube, except the final dilution, then contained $2\frac{1}{2}$ cc. of salt solution and $2\frac{1}{2}$ cc. of serum dilution, making 5 cc. in all and furnishing dilutions of serum beginning with 1:5 and ending in 1:640. To eight fresh tubes $\frac{1}{2}$ cc. of each serum dilution was now added, beginning with the highest dilution and using the same pipette up to the most concentrated solution. Finally $\frac{1}{2}$ cc. of the suspension of bacteria was added, making a series of serum dilutions beginning with 1:10 as shown in Table I. Particles in the culture suspension, such as bits of agar and clumps of bacteria, were

removed by withdrawing the suspension with a pipette through a piece of sterile cotton dropped into the stock suspension.

The distance or interval between the last tube of the series which was completely cleared by the clumping and sedimentation and the tube which showed the agglutination limit varied somewhat from case to case, as shown in Table II, which contains three illustrative examples taken from the protocols.

TABLE I.
Serum Dilution. (First Series of Tubes.)

Tube No.....	1	2	3	4	5	6	7	8
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Salt solution added.....	4	2½	2½	2½	2½	2½	2½	2½
Undiluted serum to first tube.....	1							
Diluted serum to other tubes in succession.....		→2½	→2½	→2½	→2½	→2½	→2½	→2½
Dilutions.....	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640.

Final Dilution. (Second Series of Tubes.)

Serum dilutions.....	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Amount of serum dilution added to each tube.....	½	½	½	½	½	½	½	½
Culture suspension.....	½	½	½	½	½	½	½	½
Final dilution.....	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280

This method of using at least eight final dilutions served to reveal any possible errors and control any so called paradoxical reactions. Unless the series showed a definite regular decline in agglutinins, it was done over again. By this means most paradoxical reactions were traced to some irregularity or accidental error in manipulation. Table II illustrates the protocols. Readings were made after 3 to 4 hours in the incubator, followed by refrigeration over night.

The final agglutination titer adopted was shown by that tube in which there was some definite clumping as indicated in the suspension

or amount of deposit when compared with the next lower and negative tube and the culture control. In Table II the limit is shown as 1:640 in Case 31. Nos. 19 and 101 require further dilutions to obtain the precise limit. When clumping occurred in the control the series was rejected and a fresh one prepared. The figures given in Tables III to V represent, therefore, the agglutination limit under the conditions of the test as described above.

Test for Bacillus abortus in the Udder.—The milk was collected directly into sterile glass jars during the latter part of milking. The samples averaged about 650 cc. At the laboratory the milk was transferred to sterile separatory funnels and kept over night at

TABLE II.

Illustration of the Range between Complete Sedimentation and Slight or No Clumping.

Case No.	Dilutions.								Control.
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
31	C.*	C.	C.	C.	++	++	++	—	—
101	"	"	"	"	++	+			—
					C.	++	++	++	
19	"	"	"	"	"	++	++		—
						C.	C.	++ +	

* C. indicates complete clearing of the fluid, + + + + slight clouding plus heavy deposit, and so on to —, which means no trace of clumping.

about 4–5°C. The next morning the milk was withdrawn from the funnel, leaving the cream, distributed into large centrifuge bottles, and centrifuged for 20 minutes at a speed of about 1,500 revolutions per minute. Any cream remaining was removed with a section lifter, some of the top milk withdrawn with a pipette or syphon, and the remaining milk and sediment were well mixed and distributed into smaller centrifuge tubes and centrifuged a second time for 15 minutes at a speed of about 1,400 revolutions per minute. The top milk was again withdrawn, the remaining milk and sediment were well mixed, and each sample was injected intraperitoneally into three guinea pigs. Each animal received 5 to 7 cc. Films of the milk

fresh and fixed in absolute alcohol and ether and stained with alkaline methylene blue or Giemsa's stain were examined to determine in general the character of the sediment injected. After a period of 6 to 8 weeks the guinea pigs were chloroformed and three cultures made from each animal by tearing out bits of spleen tissue and transferring to agar slants whether lesions were present or not. All cultures were sealed.

Agglutination Titer towards Bacillus abortus when the Fetus Contains Only Spirilla (Vibrio fetus).

For more detailed information concerning the bacteriological data used in the tables to follow, the reader is referred to earlier papers by Smith.^{1,9,10} Certain unpublished data were also furnished by him in individual cases to be discussed later.

In Table III are given data pertaining to nineteen cases in which spirilla were found and agglutination tests made. The remaining seven cases of the same group are omitted because blood samples were not obtained.

Taking an agglutination titer up to and including 1:40 as negative as to the presence of *Bacillus abortus*, we find that in eleven cases the agglutination titer agrees with the bacteriological findings. Of these eleven cases, the milk of three was examined for *Bacillus abortus* and found free. In two cases (Nos. 256 and 290) a maximum titer of 1:1,280 for *Bacillus abortus* was associated with the presence of this bacillus in the milk. This high titer is thus accounted for. The first four cases in the table have titers which indicate that the animals were infected with *Bacillus abortus* at some earlier date, since in three of these the milk tests were negative. The fourth was not examined. Of these No. 246 is of interest. This cow aborted May 28, 1918, with spirilla in the fetus. The fetus was small, only 7½ inches long. On September 5 and 12, blood samples registered a titer of 1:160. On February 4, 1919, a sample of milk proved free from *Bacillus abortus*. On April 30, the cow aborted again (now Case 350). *Bacillus abortus* was found in the fetus. The titer of the

⁹ Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

¹⁰ Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

blood on May 16 was 1:640. Evidently a fresh infection with *Bacillus abortus* had taken place since the previous abortion. In No. 159 there was a decline in the agglutination titer following the birth of a normal calf, asphyxiated at birth. Case 251 is the only one in the table whose titer cannot be satisfactorily explained with the data on hand. This cow aborted June 10, 1918, with positive spirilla cultures. The results were negative as to *Bacillus abortus*. On September 5, the blood titer for *Bacillus abortus* was 1:640. Two milk tests with samples, collected November 20, 1918, and March 4, 1919, were both negative. The only explanation that suggests itself is that there was associated *Bacillus abortus* with the spirilla in the fetal membranes but not in the fetus in the June abortion. The absence of any tests with uterine exudate immediately after abortion leaves this interpretation open. It is also possible that *Bacillus abortus* was in the milk in very small numbers. This case is thus the only one in the table in which the serological test does not harmonize with the bacteriological data.

One of the most instructive cases in Table III is Cow 290. The peculiarity of the agglutination titer makes a more detailed history of this case of interest. This cow gave birth to twins March 13, 1917. Both were unable to drink and too weak to stand. One was brought to the laboratory dead March 15. From Dr. Smith's notes on this case we find that there was marked fatty degeneration of the liver and extreme congestion of the medullary zone of the kidneys. The mucosa of the fourth stomach was covered with a thick viscid layer of mucus. In the lungs there were many lobules and groups of lobules collapsed. The postmortem changes made examination of the small intestines useless but the calf probably died of what is usually known as scours. Histological examination of fixed and hardened tissues showed bronchopneumonic collections of cells resembling polynuclear leucocytes, general vacuolation of liver cells, and intense congestion of the medulla of the kidneys approaching hemorrhage. Pieces of the collapsed lung tissue ground and inoculated into two guinea pigs produced the abortion disease in both cases. From these *Bacillus abortus* was isolated.

A dead fetus was discharged November 19, 1918, 24 inches long (No. 290). Spirilla were isolated in pure culture from the fourth

TABLE III.
Agglutination Test for B. abortus in Cases of Abortion Associated with Spirilla.

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for <i>B. abortus</i> in milk.	Remarks.
	<i>in.</i>								
159	16½	Purchased.	Dec. 17, 1917	3	Aug. 29, 1918	1:160			Preceding pregnancy also terminated in abortion. Etiology in doubt. Asphyxiated at birth.
(324)	Normal calf.		Feb. 12, 1919	4	Mar. 24, 1919	1:20			
192	36	Purchased.	Jan. 23, 1918	3	Aug. 29, 1918	1:80	Dec. 17, 1918	—	
213	21	"	Mar. 18, 1918	4	" 29, 1918	1:160	" 17, 1918	—	<i>B. abortus</i> found in calf.
246	7½	"	May 28, 1918	2	Sept. 5, 1918	1:160	Feb. 4, 1919	—	
(350)	Calf alive.		Apr. 30, 1919	3	" 12, 1918 May 16, 1919	1:160 1:640			
251	17½	Purchased.	June 10, 1918	2	Sept. 5, 1918	1:640	Nov. 20, 1918 Mar. 4, 1919	— —	
256	19	"	" 18, 1918	4	Oct. 3, 1918	1:1,280	Nov. 27, 1918	+	
258	27	"	" 29, 1918	2	" 3, 1918	1:20	Feb. 11, 1919	—	
267	23	"	Aug. 12, 1918	3	" 3, 1918	1:40			
269	20	"	Nov. 18, 1918	2	Nov. 21, 1918 " 28, 1918	1:20 1:20	Jan. 7, 1919	—	

stomach, large intestine, lungs, kidney, spleen, and liver. Guinea pigs inoculated with lung tissue, meconium, fourth stomach contents, and suspensions of exudate from the vagina obtained with a swab November 18 were normal when killed after 7 weeks.

On November 14, 1918, 5 days before abortion, the agglutination titer was only 1:10. 7 days later and 2 days after discharge of the fetus it was 1:1,280. The same high titer was found on November 28 and December 11. Milk drawn November 27 was found infected with *Bacillus abortus*. On April 4, 1919, the titer was still 1:1,280.

The main facts in the history of this cow are: Twins March 13, 1917, one of which carried *Bacillus abortus* in lungs. Discharge of fetus November 19, 1918, containing spirilla but not *Bacillus abortus*. Agglutination titer towards *Bacillus abortus* jumped from 1:10, 5 days before, to 1:1,280, 2 days after abortion. We have thus far been unable to find a second case with conditions paralleling this and hence are unable to present a confirmation of this somewhat unique case. Possible errors in collecting samples and in assigning the fetus to the right dam can be eliminated. The sudden rise in agglutinins may be tentatively ascribed to a rapid absorption of *Bacillus abortus* or some antigen from the udder into the circulation during discharge of the fetus. The mechanism for producing agglutinins towards *Bacillus abortus* having been established since the preceding calving, it acted promptly on stimulation by discharging large amounts of agglutinin into the blood.

No. 356 has a suspicious titer probably due to earlier *Bacillus abortus* infection. No. 351, a double infection, is discussed farther on.

In Table IV are brought together such cases of abortion as were characterized by sterile fetuses or else by miscellaneous infection of the digestive and respiratory tracts. Bacteriological details will be found elsewhere.¹

Of the eleven cases, eight show a titer of 1:20 or lower. One (No. 211) with a high titer of 1:640 is explained by an earlier *Bacillus abortus* infection and by a demonstration of *Bacillus abortus* in the milk. The first sample of milk was negative, the second positive. In another (No. 259) with a titer of 1:160 this may be regarded as a remnant of the preceding pregnancy which terminated in abortion associated with *Bacillus abortus*. The same may be true of a third

TABLE IV.
Agglutination Titer in Cases of Abortion with Sterile Fetus or Miscellaneous Infection.

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for <i>B. abortus</i> in milk.	Remarks.
	in.		1917		1918		1919		
103	4	Native.	June 24	3	Oct. 30	1:10			Normal births Aug. 8, 1918, and Mar. 26, 1919.
122	24	Purchased.	July 31	1	Nov. 14	0			Normal calf Nov. 16, 1918.
126	(75 lbs.)	"	Aug. 15	2	" 14	1:20			" " 19, 1918.
143	(35 ")	"	Oct. 11	1	Oct. 30	1:20	Jan. 10	—	" " Oct. 17, 1918.
201	34	"	Feb. 12	2	" 9	1:10	" 10	—	Preceding pregnancy terminated Apr. 4, 1917, by <i>B. abortus</i> infection.
211	23	Native.	Mar. 13	4	Sept. 27	1:640	" 10	—	
					1919		Apr. 11	+	Normal calf Apr. 4, 1919.
259	20	"	July 18	2	Apr. 26 June 3 1918	1:1,280 1:2,560	Jan. 2	—	Preceding pregnancy infected with <i>B. abortus</i> ; present one with <i>B. pyogenes</i> .
262	29	Purchased.	" 28	1	" 3	1:20			Aborted Jan. 20, 1918. No bacteriological data.
279	8	Native.	Sept. 27	2	" 3	1:320			Infection with <i>B. pyogenes</i> .
291	32	"	Nov. 19	4	Nov. 28	1:10	Jan. 2	—	
300	35	Purchased.	Dec. 7	6	Dec. 11	1:20			

TABLE V.
Agglutination Reaction of Cows, Native and Purchased, from Which B. abortus Was Isolated.

Case No.	Length of fetus. <i>in.</i>	Native or purchased cow.	Date of abortion.	No. of preg-nancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for <i>B. abortus</i> in milk.	Remarks.
17	27	Native.	Mar. 9	1	Nov. 7	1:40	1918		Normal calf Apr. 14, 1918.
26		"	" 27	1	" 21	1:20			" calves Jan. 17, 1918, and Mar. 22, 1919.
31	27	Purchased.	Apr. 5	1	" 28	1:640	Dec. 17 1919	+	Normal calf Oct. 3, 1918.
33	26	Native.	" 6	1	" 21	1:640	Feb. 18	+	" June 8, 1918.
34*	Living calf.	"	Feb. 23		" 21	1:320	" 20	+	" calves Feb. 23, 1917, and Mar. 3, 1918. Cow aborted Nov. 10, 1918. No bacteriological examination.
41	Nearly mature.	"	Apr. 22	1	" 14	1:40	" 20	+	Normal calf Oct. 17, 1918.
43	(8 mos.)	"	" 23	1	" 21	1:640	" 20	+	" Apr. 23, 1918.
86	21½	"	May 16	1	" 14	0			" Oct. 25, 1918.
89	Alive.	"	" 16	1	" 21	1:40			" Aug. 6, 1918.
91	30	"	" 5	1	" 21	1:80			" " 1, 1918.
101*	Normal calf.	Purchased. Native.	June 28	1	Oct. 30	1:1,280	Feb. 28	+	Placenta appears normal. Normal calf June 9, 1918.
128	Calf dies (scours).	Purchased.	Aug. 17	1	" 20	1:10			<i>B. abortus</i> isolated from calf's intestine.
134	Calf alive.	Native.	Sept. 4	1	Nov. 14	1:320	Feb. 18	+	Normal calf Sept. 26, 1918. <i>B. abortus</i> obtained from placenta.
									Normal calf Nov. 8, 1918.

144*	Normal calf.	Purchased.	Oct. 8	1	Oct. 30	1: 640	Mar. 4	+	Normal calf Mar. 21, 1919.
164	Alive.	Native.	Nov. 20	1	" 9	1: 320	" 4	+	7 wks. premature.
188*	Calf alive.	Purchased.	Dec. 31	1	Jan. 2	1: 1,280	Feb. 26	-	
200	21	Native.	Feb. 12	1	Oct. 17	1: 640	" 26	+	Calf very sick with scours when killed.
203	Calf alive.	"	" 15	1	Sept. 27	1: 640			
206	28	"	" 24	1	" 27	1: 160			
210	Calf alive.	"	Mar. 6	1	" 27	1: 40			
214	"	Purchased.	" 15	1	" 27	1: 320			
215	"	Native.	" 11	1	" 18	1: 1,280			
220*	"	"	" 30	1	" 18	1: 640	Nov. 12	+	Multiple staphylococcus abscesses in lung.
222	"	Purchased.	Apr. 11	1	" 18	1: 1,280			
224	"	Native.	" 12	1	" 18	1: 640			
226	"	"	" 12	2	" 12	1: 1,280			
227	"	"	" 15	1	" 12	1: 640			
253	25	Purchased.	June 13	2	" 27	1: 160			
266	31	Native.	Aug. 5	3	Oct. 3	1: 320	Nov. 20	+	
270	29½	Purchased.	" 22	2	Sept. 5	1: 640	1919		
273	16	"	" 27	2	" 5	1: 1,280	Jan. 11	-	
278	14½	"	Sept. 19	1	Oct. 4	1: 40			
281	30½	"	Oct. 4	1	" 9	1: 160			
285	28½	Native.	" 23	1	Jan. 2	1: 640			
288*	Calf alive.	Purchased.	Nov. 17	1	" 2	1: 1,280	Feb. 4	+	Calf has scours.

* Included because the milk was examined. *B. abortus* was not isolated from the calf.

TABLE V—Concluded.

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for <i>B. abortus</i> in milk.	Remarks.
	<i>in.</i>		1918		1918		1918		
298	34	Native.	Dec. 2	1	Dec. 11	1:80			
301	Calf alive.	"	" 11	2	Nov. 28	1:1,280	Dec. 10	+	<i>B. abortus</i> isolated from uterine exudate. <i>B. abortus</i> found in fetus expelled Mar. 20, 1917.
			1919		1919				
309	(58 lbs.)	Purchased.	Jan. 2	1	Jan. 8	1:320			
311	(38 ")	"	" 7	1	" 16	1:640			
313	29	Native.	" 12	4	" 23	1:640			
316	22½	"	" 18	2	" 18	1:1,280			
328	31	"	Mar. 9	2	Mar. 24	1:640			No record of former abortions.
329	30	Purchased.	" 11	1	" 24	1:640			
350	Calf alive.	"	Apr. 30	3	May 16	1:640			Aborted in preceding pregnancy.
351	28	"	May 5	1	" 15	1:20			Fetus contains both spirilla and <i>B. abortus</i> .
					June 2	1:160			

case (No. 279). Unfortunately a milk sample from this cow was not obtainable.

In Table V all cases of abortion associated with *Bacillus abortus* in the fetus or membranes or in the udder are brought together. The agglutination limits as given easily bring the cases into several categories. Taking those cases in which the serum clumps *Bacillus abortus* in dilutions of 1:20 and 1:40, but not up to 1:80, we find, out of 44, 8 in this class. The low titer in certain cases (Nos. 17, 26, 41, 86, 89, and 128) may be referred to the long period between abortion and the blood test, ranging from 14 to 20 months. In No. 210, the low titer of 1:40 may be due to a mild, possibly quite recent infection of the fetal membranes. The calf was vigorous and apparently in normal condition. No. 278 with a titer of 1:40 discharged a macerated fetus about 3½ months old. Here also the infection may have been too recent to stimulate antibodies.

No. 351 is of interest since both *Vibrio fetus* and *Bacillus abortus* were isolated from the fetus. The dam was well along (6½ to 7 months) in pregnancy. *Bacillus abortus* infection may have been superinduced late on the spirillum infection, since the agglutination titer for *Bacillus abortus* rose after abortion.

There are but two cases with a titer of 1:80. In one (No. 91) this was determined 18 months after abortion and may have fallen from a higher level. The second (No. 298) cannot be explained in the same way since the blood test was made 9 days after abortion. In this case, however, the fetus was large and the duration of pregnancy over 7 months. Here also the immune reaction of the dam may not have reached its maximum owing to late infection.

The two cases with a titer of 1:160 are probably explainable in much the same way as the two preceding ones. In one the blood test was made 5 days, in the other 7 months after abortion. Both fetuses were large.

Taking the next higher titer, 1:320, we find six on this level. Nos. 134 and 164 carried *Bacillus abortus* in the milk according to recent tests. How far back this milk infection dates cannot be determined. Most likely it started with the abortion indicated in the table. In the case of Nos. 214, 253, 273, and 309 the longest period between the discharge of fetus or calf and the agglutination test was less than 7 months.

The next highest titer, 1:640, was held by sixteen cases. In the two earliest cases recorded (Nos. 31 and 33) the blood test followed abortion by 19 months. The titer should have been low after such a long period but the udder was infected. In the next case (No. 144) the blood test followed abortion by nearly 13 months. In this case also the high titer is explainable by the udder infection. In the remaining thirteen cases the high titer is amply accounted for by the recent abortion. The milk, tested in only three of these, was found infected in all.

The highest recorded titer in this group, 1:1,280, was held by nine cases. The agglutination test in No. 101 is referable to the milk infection. No. 188 is an unexplained case. The test for *Bacillus abortus* in the milk was negative. A second sample is now being examined.¹¹ The other cases may be explained by the presence of *Bacillus abortus* in the fetus. No. 270 is of interest in that a high titer was present 14 days after abortion, although the sample of milk tested subsequently was free from infection.

SUMMARY.

The agglutination test when carried out so as to give the entire range of serum dilutions to the limit of clumping is a delicate test which reflects a variety of conditions involved in infection with *Bacillus abortus*. Among these conditions are its time relation to the act of abortion and the length of time the abortion bacilli live and multiply in the pregnant uterus. It is obvious that if a uterus be infected in the 8th month of pregnancy, the opportunity for agglutinins to accumulate are poorer than if the uterine infection lasts 3 or 4 months. The presence of *Bacillus abortus* in the udder determines in many cases the intensity of the reaction. No definite rules can therefore be formulated for the interpretation of the agglutination reaction quantitatively, since it is bound up with a complicated process varying from case to case. In the individual cow in general a titer of 1:40 or less may be regarded as indicating that the cow is not infected with *Bacillus abortus* at the time of the blood examination. It does not exclude former infections in the case of older cows, nor does it absolutely exclude very recent infection (Nos. 278 and 351).

¹¹ The second test of the milk of No. 188 was negative.

The highest titers, 1:640 and above, generally indicate recent infection and in the absence of recent premature births infection of the udder. Even when abortion has just occurred, it may be due to other agencies and the high titer maintained by a chronic infection of the udder dating from an earlier uterine infection with *Bacillus abortus*. Intermediate titers may indicate a gradual rise or decline of agglutinins preceding or following abortion without infection of the udder. They may also stand for a relatively high resistance or partial immunity of the cow.

In any herd a uniformly low titer (1:40 or less) in all animals may be regarded as indicating the entire absence of *Bacillus abortus*. A high titer in any one cow serves to indicate quite definitely the presence of infection in the herd. To determine more accurately the character of the infection in any individual cow there is needed in addition to the quantitative agglutination test a bacteriological study of the milk and of any prematurely discharged calf or fetus.

HYDROGEN ION CONCENTRATION OF CULTURES OF PNEUMOCOCCI OF THE DIFFERENT TYPES IN CARBOHYDRATE MEDIA.

By O. T. AVERY, M.D., AND GLENN E. CULLEN, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, July 1, 1919.)

Knowledge of the physiological activities and immunological characters of bacteria serves not merely the purposes of systematic classification, but contributes to a fuller understanding of the problems of infectious disease. The correlation of these apparently independent characters with pathogenicity and with the occurrence and distribution of recognizable types under a wide variety of environmental conditions is essential to the proper interpretation of the phenomena of infection.

Of the physiological characters of bacteria the fermentation of certain carbohydrates by some, and the inability of other closely related organisms to utilize the same substances, are relatively fixed characteristics of sufficient constancy to warrant their use as a basis for differentiation. In fact, within a given species these biochemical reactions are of considerable value in distinguishing type relationships. The application of this principle in the classification of the typhoid-dysentery group affords an illustration of the value of the biochemical method in determining the existence of certain varieties of these organisms and their relation to enteric infections. On the other hand, it has been possible in the case of pneumococcus to acquire knowledge of the occurrence of specific types and their relation to disease, chiefly by a study of the immunological characters of these organisms, since their biochemical activities are apparently possessed in common by all.

The measure of acid production by bacteria has been the determination of the amount of titrable acidity produced in a medium containing a known concentration of carbohydrate. This method is now recognized as inadequate. Recently the colorimetric determination

of the hydrogen ion concentration has been adapted to bacteriological requirements and affords an additional method for measuring this phase of bacterial metabolism. Moreover, in addition to the selective fermentation of certain substances by bacteria, the maximum acidity that can be tolerated by a given organism is apparently a biologic constant. Accordingly, the determination of the hydrogen ion concentration at which bacterial growth ceases has been utilized as a differential method. In the case of a culture growing in a medium containing an excess of fermentable substance this limiting reaction is spoken of as the final hydrogen ion concentration. This phenomenon has been utilized by Clark (1) and Clark and Lubs (2) in the differentiation of the colon-*aerogenes* group, and by Ayers (3), Ayers, Johnson, and Davis (4), and Avery and Cullen (5) in the recognition of differences between hemolytic streptococci of human and bovine origin.

The present paper presents the results, obtained under the experimental conditions defined, of a study of the influence of the concentration of dextrose on the final hydrogen ion concentration of broth cultures of pneumococcus, the rate of acid production, the optimum and limiting hydrogen ion concentration for initial growth of pneumococcus, the final hydrogen ion concentration of cultures of pneumococcus in carbohydrate media, and a comparison of these physiological functions with the specificity of the immunological type characters of these organisms. In addition, some information has been acquired concerning the factors governing growth of pneumococcus when reinoculated into filtrates of cultures of the same and different types.

EXPERIMENTAL.

Source of Material.—Thirty-nine strains of pneumococcus comprising representatives of the various immunological types were studied. In the majority of instances these cultures were isolated directly from the blood or sputum of patients suffering from lobar pneumonia. Some of the strains were tested immediately upon isolation, others after years of cultivation on artificial media. Most of the strains were pathogenic for white mice and had been passed through these animals to enhance virulence. Cultures used for inoculation of the test medium were grown for 18 hours at 37°C. in plain meat infusion broth of pH

7.8. The serological methods used for the determination of types of pneumococcus were the same as those already described (6).

Determination of Hydrogen Ion Concentration.—The colorimetric method of determining hydrogen ion concentration was chosen because of its convenience and simplicity. The principle and details of this method have been so thoroughly reviewed that it is unnecessary to repeat them here (7). In the present work the two principal sources of error, color of the medium and turbidity of the culture, have been avoided by first diluting the medium and then compensating for the color by Walpole's comparator method of superimposing the color of the medium upon that of the indicator.

The hydrogen ion concentrations are expressed in the customary manner as pH values; that is, the negative exponent to the base 10 of the normality.

Duplicate tubes containing 5 cc. of the medium or culture were diluted to 15 cc. with redistilled water, four drops of indicator solution were added to one tube, and the tube was compared in a comparator block with a standard solution containing exactly the same amount of indicator. The standard solutions of known hydrogen ion concentration were prepared for the range pH 8.6 to 5 from standard phosphates by Sørensen's technique, and for the range pH 5.8 to 4.6 from acetic acid-sodium acetate mixtures by Walpole's directions. The accuracy of these standard solutions was verified by the hydrogen electrode.

The indicators used were:

	<i>per cent</i>
pH 8.6-8.0, <i>o</i> -cresol sulfonephthalein.....	0.04
pH 8.0-6.8, phenolsulfonephthalein (phenol red).....	0.04
pH 6.8-5.8, bromocresolsulfonephthalein, saturated water solution.	
pH 5.8-4.6, methyl red.....	0.04

Sterilization of the Medium Containing the Test Substance.—In studying fermentative activity of bacteria the manner in which the medium containing the test substance is sterilized is of first importance. If a sugar, such as sucrose, is added to the slightly alkaline broth and sterilized in an Arnold sterilizer or boiled for a long time, the sugar will be hydrolyzed. It seemed best, therefore, to sterilize by boiling a concentrated solution of the substance in water. Enough of this con-

centrated solution is then added to sterile broth to the desired dilution. 10 minutes in boiling water has sufficed in most cases to effect sterilization. When the medium has been handled many times, in adjusting to different reactions, further sterilization has been considered necessary. Additional sterilization is indicated in the protocol.

Influence of Sugar Concentration upon the Final Hydrogen Ion Concentration.—In studying the sugar-fermenting property of any organism it is, of course, essential to determine the influence of varying concentrations of sugar upon the final reaction. In order to do this for pneumococcus the following experiment was carried out.

To sugar-free broth of known hydrogen ion concentration sterile 20 per cent dextrose solutions (boiled 10 minutes) were added in sufficient amounts to make the desired concentration. The medium was incubated over night to test sterility, and 50 cc. portions were inoculated with 0.1 cc. of an 18 hour plain broth culture of pneumococcus. The hydrogen ion concentration of the medium was determined before the inoculation and after 24 and 48 hours incubation (Table I).

TABLE I.

Influence of the Concentration of Dextrose on the Final Hydrogen Ion Concentration of Pneumococcus Cultures.

50 cc. of sugar-free* broth, pH 7.5, inoculated with 0.1 cc. of 18 hour plain broth culture of *Pneumococcus* Type II (Strain F 149).

Dextrose.	Hydrogen ion concentration.	
	24 hrs.	48 hrs.
<i>per cent</i>	<i>pH</i>	<i>pH</i>
Broth control (uninoculated).	7.5	7.5
0	7.3	7.2
0.1	6.5	6.5
0.2	6.0	6.0
0.4	5.1	5.0
1.0	5.0	5.0
2.0	5.0	5.0
4.0	5.0	4.9

* In the preparation of sugar-free broth the meat infusion, before the addition of peptone, is fermented by *B. coli* for 18 to 24 hours.

It is evident that 0.4 per cent dextrose furnished sufficient acid to bring the medium to the limiting hydrogen ion concentration, but that excess of sugar up to at least 4 per cent has no influence upon the final reaction. 1 per cent, therefore, was chosen as sufficient for all routine fermentations. This agrees closely with a similar experiment with *Streptococcus hemolyticus* (5).

Rate of Acid Production of Pneumococcus in 1 Per Cent Dextrose Broth.—It was to be expected that the rate of acid production would be dependent upon the size of the inoculum, but it was desirable to compare the rate curve in dextrose broth with that in plain broth. Moreover, it was necessary to determine the time required for the attainment of the final hydrogen ion concentration. In determining the rate a massive inoculum was used to bring the experiment within 1 day (Table II).

TABLE II.

Rate of Acid Production by Pneumococcus in Dextrose Broth.

100 cc. of sugar-free broth plus dextrose to 1 per cent, inoculated with 0.5 cc. of an 18 hour plain broth culture of pneumococcus, and incubated at 37°C.

Pneumococcus.	1 per cent dextrose broth. Initial hydrogen ion concentration.	Hydrogen ion concentration.					
		1½ hrs.	4 hrs.	6 hrs.	8 hrs.	9 hrs.	27 hrs.
	pH	pH	pH	pH	pH	pH	pH
Type I	7.75	7.7	7.3	6.5	5.6	5.3	5.0
“ II	7.9	7.9	7.5	6.8	5.7	5.3	4.8

No change in reaction of the dextrose broth occurred during the period of initial lag, then acid was produced at a rapid and constant rate until the final reaction was reached—in this case a pH 4.8 to 5.

With the usual inoculum employed in these experiments, 0.1 cc. of an 18 hour culture to 50 cc. of broth, the final hydrogen ion concentration is attained within 24 hours. An occasional culture will show a further increase in acidity of not more than 0.1 pH after 2 to 7 days incubation, but such changes are within the limits of experimental error. Table III furnishes an illustration of this fact. In a few instances in this experiment 18 hours was not sufficient time for the

attainment of the final reaction, but no apparent changes took place after 30 hours. As a routine, therefore, the cultures were read after 24 or 48 hours incubation at 37°C.

TABLE III.

Relation of the Type of Pneumococcus to the Final Hydrogen Ion Concentration in Sugar Broth.

75 cc. portions of 1 per cent sugar broth having an initial reaction of pH 7.8 were inoculated with 0.1 cc. each of an 18 hour plain broth culture of pneumococcus of different types.

Pneumococcus.		Hydrogen ion concentration.					
Type.	Strain.	Dextrose.			Lactose.		
		18 hrs.	30 hrs.	5 days.	18 hrs.	30 hrs.	5 days.
		pH	pH	pH	pH	pH	pH
I	F 55	5.1	5.1	5.1	5.1	5.1	5.1
I	146	5.0	5.1	5.0	5.0	5.0	5.0
II	46	4.9	5.0	5.0	5.0	5.0	5.0
II	F 149	5.2		5.1	5.1	5.1	5.1
IIa	J	5.8	5.0	5.0	5.3	4.9+	5.0
IIb	W	5.1	5.2	5.2	5.3	5.1	5.1
III	A 66	5.1	5.05	5.05	5.1	5.0	5.1
III	F 104	5.0	5.0	5.0	5.1	5.0	5.1
IV	L F	5.0	5.0	4.9	5.1	5.2	5.1
IV	L A	5.0	5.2	5.2	5.1	5.2	5.2

Relation of the Type of Pneumococcus to the Final Hydrogen Ion Concentration of Cultures in Sugar Broth.—In order to determine whether the differences in antigenic properties of the specific types of pneumococcus had any influence on the final reaction in sugar medium, two strains of each of the four types, as well as strains representing subgroups of Type II were inoculated into broth containing 1 per cent dextrose or lactose. It is evident from Table III that the final pH of all strains of pneumococcus was between pH 5.2 and 4.9, with the majority between pH 5.0 and 5.2. The differential characters as determined by immunological reactions were not evident from the final hydrogen ion concentrations. It is of significance that the final hydrogen ion concentration of cultures of pneumococcus is exactly the same as the final reaction of pathogenic hemolytic streptococci of

human type. This is also brought out in Table IV where the results of all the strains used in these and succeeding experiments are summarized by types. This table represents 54 determinations on 39 strains. Duplicate determinations in individual experiments are not included in this table.

TABLE IV.

Final Hydrogen Ion Concentration of Pneumococcus in Dextrose Broth. Summary of 54 Determinations on 39 Strains.*

Type I.		Type II.		Type III.		Type IV.	
Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.
	pH		pH		pH		pH
I	5.2	II	5.0	A 66	5.0	L F	5.0
I	5.0	II	4.9	A 66	5.1	L A	5.2
I	5.1	F 149	5.1	F 104	5.0	F 194	5.1
F 55	5.1	F 149	5.1	F 104	4.8	X 47	5.2
E 22	5.1	F 149	5.1	D 40	5.0	E 190	5.0
F 152	5.0	F 149	5.1	D EH	4.9	E 190	4.8
D 6	5.0	F 149	5.0	U	5.1	E 117	5.0
D 46	5.0	F 149	5.0	E 84	5.0	E 157	5.0
D 46	4.8	F 149	5.1	C 28	5.0	E 121	5.2
D 46	5.0	F. 149	5.3	E 111	4.9	D 107	5.0
16,887	5.0	D 39	5.0	G	5.0	Sch.	4.9
16,867	5.0	IIa J	5.0	E 127	5.0		
F 169	5.0	IIb W	5.0	A	4.9		
		IIc A	5.0				
		IIc 50	5.0				
		II atypical, F 150	5.0				
		II " F 154	5.1				
Total	13	17		13		11	
Average.	5.0		5.0		4.9		5.0

* Repetition of strains in the above table represents observations made in different experiments.

It is evident from the preceding experiment that with dextrose and lactose there is no type difference in acid production. A number of other carbohydrates fermentable by pneumococcus have been tested.¹

¹ The substances used were either Kahlbaum's reagents or were those prepared by Mr. E. P. Clark of The Rockefeller Institute for Medical Research.

The media were prepared by placing solutions or suspensions of the substance in boiling water for 10 minutes, and adding the required amount to 75 cc. of media. The media were then heated in the Arnold sterilizer once for 20 minutes, and incubated to test sterility. They were inoculated with 0.1 cc. each of 18 hour plain broth cultures of pneumococcus representing the four types. Samples were removed for pH determinations after 48 hours and 7 days. Since 7 day readings are practically identical with 48 hour readings, only the latter are given (Table V).

TABLE V.

Final Hydrogen Ion Concentration of Pneumococcus in Various Carbohydrate Media.

75 cc. of sugar-free broth containing 1 per cent carbohydrate, initial pH 7.5, were inoculated with 0.1 cc. of an 18 hour plain broth culture and incubated 48 hours at 37°C.

Test substance.	Uninoculated control.	Pneumococcus type.			
		I (Strain F 152).	II (Strain F 149).	III (Strain F 104).	IV (Strain E 190).
	pH	pH	pH	pH	pH
Maltose.....	7.5	5.0	5.0	4.9	5.0
Saccharose.....	7.5	5.0	5.1	5.0	5.0
Lactose.....	7.5	5.0	5.0	5.0	4.9
Galactose.....	7.3	5.1	5.1	5.1	4.8
Raffinose.....	7.5	5.0	5.1	5.1	5.1
Dextrose.....	7.5	5.1	5.1	4.8	4.8
Inulin.....	7.5	5.1		5.1	5.1

From the results presented in Table V it is evident that in media containing sufficient fermentable carbohydrate, growth of pneumococcus continues until a final hydrogen ion concentration of about pH 5 is reached. Apparently this acidity is sufficient in itself to stop growth and the organisms die in the products of their own metabolism. Cultures of pneumococci with all the carbohydrates which were fermentable under the conditions used, namely maltose, saccharose, lactose, galactose, raffinose, dextrose, and inulin, gave identical results in the rate of reaction change and final hydrogen ion concentration (pH 5) attained. Further, the different immunological types of pneumococcus, in a limited number of strains studied, behave alike in fermenting these carbohydrates.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus.—Work previously reported from this laboratory (8) showed that the optimum hydrogen ion concentration for the growth of pneumococcus was about pH 7.8, and that the hydrogen ion concentrations within which growth could be initiated were between pH 7.0 and 8.3. It was desirable to determine whether the optimum pH in sugar broth was the same as that in plain broth and also to determine for dextrose broth the upper and lower hydrogen ion concentrations beyond which initial growth does not occur.

50 cc. portions of plain broth containing 0.2 per cent phosphate and the same broth containing 1 per cent dextrose were adjusted to the desired reactions by addition of either sodium hydroxide or hydrochloric acid. The media were placed in boiling water for 10 minutes, and incubated for 48 hours to test sterility. They were then inoculated with 0.25 cc. of an 18 hour culture of *Pneumococcus* Type I (Strain F 169). After 6 hours, estimation of growth and pH determinations were made, and a portion was autoclaved for determination of turbidity by the nephelometer method (Kober instrument). Essentially the same procedure was used as that described by Dernby and Avery (8), except that it seemed unnecessary to estimate the number of organisms per cubic centimeter, since it is in reality a function of the size of the inoculum. The readings on the nephelometer were expressed, therefore, as percentages of the heaviest growth (Table VI).

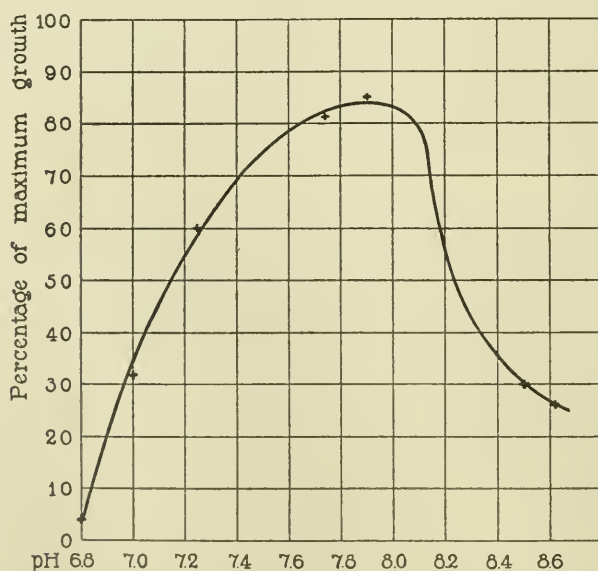
This method is entirely satisfactory with plain broth, and the results are in agreement with those of Dernby and Avery (8); it is beset with some difficulty, however, in the case of sugar broth. With plain broth the experiment may be continued to the maximum growth possible, but in sugar broth the readings must be made before acid precipitation takes place. Slight variations in reading and in initial lag are therefore emphasized. However, the curve constructed from the results was, with one exception, smooth (Text-fig. 1). The experiment was repeated with the same general result but with discrepancies in individual tubes. Another experiment was then planned, in which in addition to the turbidity method, pH determinations were made at regular intervals in order to give an idea of the time required to reach a given pH.

TABLE VI.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus.

50 cc. portions of plain broth and 1 per cent dextrose broth were adjusted to the indicated hydrogen ion concentration and inoculated with 0.25 cc. of *Pneumococcus* Type I (Strain F 169). Readings were made after 6 hours at 37°C.

Initial hydrogen ion concentration of media.	Plain broth.		Dextrose broth.	
	Turbidity.	Hydrogen ion concentration.	Turbidity.	Hydrogen ion concentration.
<i>pH</i>	<i>per cent</i>	<i>pH</i>	<i>per cent</i>	<i>pH</i>
8.4	23	8.1	80	7.5
8.3	70	7.5		
8.0	73	7.4	100	7.2
7.8	65	7.1	37	7.5
7.5	39	7.1	39	7.1
7.3		6.9	16	7.1
7.0		6.9		6.9
6.5				6.5
6.0				6.0
5.5				5.5



TEXT-FIG. 1. Effect of initial hydrogen ion concentration on growth of pneumococcus in dextrose broth.

100 cc. portions of plain broth containing 1 per cent dextrose were adjusted to the desired pH, and 25 cc. removed for controls. After 24 hours at 37°C. as a sterility test, the 75 cc. were inoculated with 0.5 cc. of a 12 hour broth culture of *Pneumococcus* Type I-1 (this heavy dose was given in order to complete the experiment within 1 day). At intervals 5 cc. samples were removed for pH determinations, and when these readings indicated active growth 5 cc. samples were autoclaved for nephelometer readings. Macroscopic estimations of growth were also made to a +++ scale. The results are given in Table VII and in Text-fig. 2.

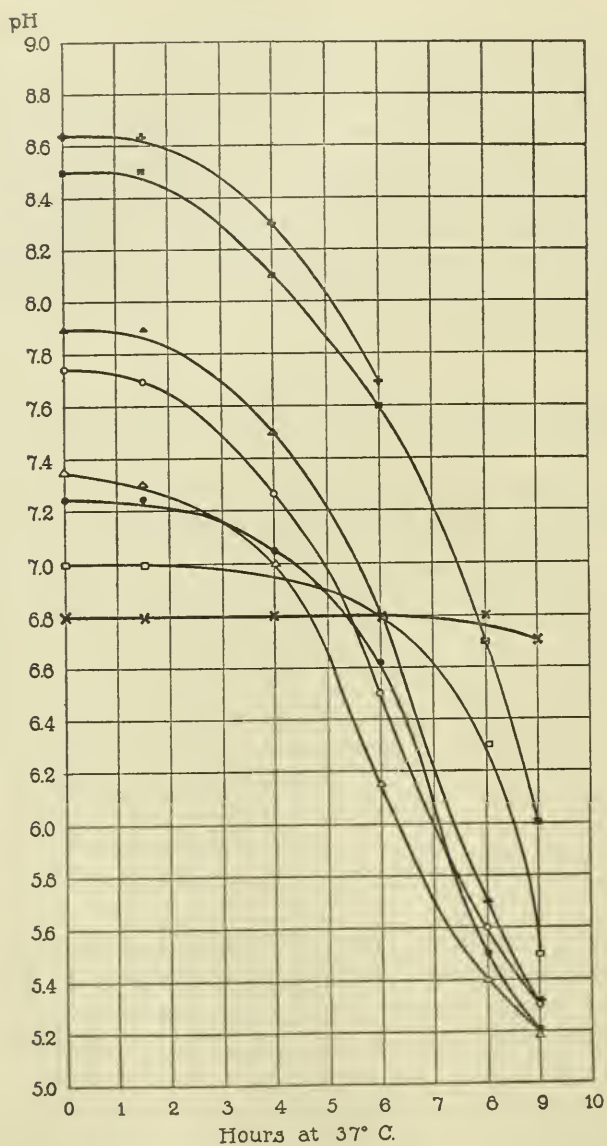
TABLE VII.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus in Dextrose Broth.

75 cc. of plain broth containing 1 per cent dextrose were inoculated with 0.5 cc. of a 12 hour broth culture of *Pneumococcus* Type I-1.

Hydrogen ion concentration.						Growth.		Turbidity. 6 hrs.
Control.	1½ hrs.	4 hrs.	6 hrs.	8 hrs.	9 hrs.	1 hr.	6 hrs.	
pH	pH	pH	pH	pH	pH			per cent
6.8	6.8	6.8	6.8	6.8	6.7	—	±	4.2
7.0	7.0	7.0	6.8	6.3	5.5	—	+	32.0
7.25	7.25	7.05	6.6+	5.5	5.2	+	+++	60.0
7.35	7.3	7.0	6.15	5.4	5.2	+	+++	
7.75	7.7	7.3	6.5	5.6	5.3	+	+++	82.0
7.9	7.9	7.5	6.8	5.7	5.3	—	+++	85.0
8.5	8.5	8.1	7.6	6.7	6.0	—	+±—	30.0
8.6+	8.6+	8.3	7.7			—	+	26.0

It is evident from these experiments that the optimum hydrogen ion concentration for growth of pneumococcus in dextrose broth was about pH 7.8; that is, the same as for plain broth (Text-fig. 1). Cultures grow luxuriantly between pH 7.2 and 8.6. The upper limit for growth in sugar medium is somewhat higher than in plain medium. Although fermentation in sugar broth starts at a pH of 7.8 and proceeds until a pH of 5 is reached, it is not possible to initiate growth below pH 7.0. The curve of the rate of acid production for this series has been also constructed (Text-fig. 2). At pH 7.75 to 7.3 the initial lag is decreased, but in all the tests from pH 7.2 to 8.6 after the initial



TEXT-FIG. 2. Effect of initial hydrogen ion concentration upon the rate of reaction change due to growth of pneumococcus in dextrose broth.

lag luxuriant growth occurs at about the same rate. This experiment emphasizes the fact that the term limiting hydrogen ion concentration must be carefully defined and that the final hydrogen ion concentration may be entirely different from the hydrogen ion concentration limits within which growth may be initiated.

Limiting Initial Hydrogen Ion Concentration.—It is brought out in the preceding experiments that the ordinary inoculation of pneumococcus fails to initiate growth if the hydrogen ion concentration of the medium is greater than pH 7.0, but that with growth well started at a pH above 7.0, acid production proceeds in sugar media to a final pH of 5.0. It would seem possible then that either the bacteria rapidly adapt themselves to the changing and hitherto unfavorable reaction, or that the medium itself is rendered more suitable for growth.

If the explanation lies in rapid adaptation of the organism one would expect that bacteria removed from a sugar broth at a reaction of pH 6.0, for example, could grow in fresh broth of the same reaction. An attempt to test this point was carried out as follows:

Dextrose broth was inoculated with 0.5 cc. of an 18 hour culture of *Pneumococcus* Type I. The change in reaction was followed, and at pH of 7.1, 6.5, 5.5, and 5.0 fresh samples of dextrose broth of varying hydrogen ion concentrations were inoculated with the actively growing culture. This broth had previously been adjusted to the desired reaction, tubed in 5 cc. portions, and incubated to test sterility. The heavy inoculation of 0.1 cc. of culture per 5 cc. of medium was used, and in addition several tubes were inoculated with the massive dose of 0.5 cc. of culture to 5 cc. of medium. The 13 hour culture (pH 5) was still viable as evidenced by the occurrence of growth when inoculated into fresh broth of pH 7.8. Only traces of disintegration of the cells were visible under the microscope. This experiment is recorded in Table VIII.

Study of Table VIII shows that it is impossible to initiate growth in media having a more acid reaction than pH 6.8 regardless of the fact that the inoculum may have been removed from an actively growing culture at ranges of acidity from pH 7.1 to 5.0. For instance, if the organisms are alive and growing at a pH 5.5, and a seeding is removed at this point and implanted in medium with a reaction of pH 6.5, no growth occurs.

TABLE VIII.

Failure of Actively Growing Cultures at pH below 6.8 to Initiate Growth in Fresh Broth of pH below 6.8.

5 cc. of dextrose broth of the indicated reaction were incubated for 24 hours at 37°C. after inoculation with cultures which had developed an acid reaction.

Culture (Type I).		1 per cent dextrose broth.	Inoculation per 5 cc.			
Age.	Hydrogen ion concen- tration.		0.1 cc.		0.5 cc.	
			Growth.	Final hydrogen ion concen- tration.	Growth.	Final hydrogen ion concen- tration.
<i>hrs.</i>	<i>pH</i>	<i>pH</i>		<i>pH</i>		<i>pH</i>
6½	7.1	7.8	++++	4.9		
8	6.5		++++	4.8		
9	5.5		++++	4.9		
13	5.0		++++	4.9		
6½	7.1	7.0	—	7.0—		
8	6.5		—	6.8+	++++	4.9
9	5.5		++++	5.0	++++	5.0
13	5.0		++++			
6½	7.1	6.8	—	6.8		
8	6.5		++++	4.9	++++	4.9
9	5.5		—	6.5	++++	4.9
13	5.0		—	6.8		
6½	7.1	6.5	—	6.5		
8	6.5		—	6.3	—	6.1
9	5.5		—	6.35	—	6.1
13	5.0		—	6.4		
6½	7.1	6.0	—	6.0		
8	6.5		—	6.0	—	5.9
9	5.5		—	5.95	—	5.8
13	5.0		—		—	5.8

Growth of Pneumococcus in Filtrates of Dextrose Broth Cultures of Pneumococcus.—An attempt was next made to determine whether dextrose broth in which pneumococcus has grown to the final hydrogen ion concentration will sustain growth if the organisms are removed by Berkefeld filtration and the reaction of the filtrate is readjusted to ranges of acidity from pH 7.8 to 6.0.

To salt-free broth 0.2 per cent sodium phosphate and 1 per cent dextrose were added, the medium was adjusted to a pH 7.8, and inoculated with Type I pneumococcus. After 24 hours incubation the culture was filtered through a Berkefeld filter. Samples of the filtrate, the pH of which was 5.2, were adjusted with sodium hydroxide to pH 6.0, 6.5, 6.8, 7.0, and 8.0; 5 cc. of filtrate required 3.3 cc. of 0.05 N sodium hydroxide to bring it to a pH 8.0. The filtrate at each pH was divided into 5 cc. portions, one of which was used as a test for sterility. Tubes containing 5 cc. of filtrate were inoculated in duplicate with 0.1 cc. of 18 hour cultures of Types I and II. The results are given in Table IX.

TABLE IX.

Growth of Pneumococcus in Filtrates of Dextrose Broth Cultures of Pneumococcus.

Dextrose broth pH 7.8. After 24 hours the culture was filtered through a Berkefeld filter. The pH of the filtrate was 5.2.

Filtrate from Type I (Strain 183) adjusted to.	48 hrs. after inoculation with.			
	Type I (Strain 183).		Type II (Strain D 39).	
	Growth.	Hydrogen ion concentration.	Growth.	Hydrogen ion concentration.
pH		pH		pH
8.0	++++	5.3		
7.0	++++	5.2	++++	5.1
6.8	++++	5.2	++++	5.2
6.5	—	6.5	—	6.5
6.0	—	6.0	—	6.0

It is evident that filtrates of dextrose broth in which pneumococcus has grown and the reaction of which has been readjusted with sodium hydroxide will not allow growth to be initiated if the readjusted pH is below 6.8.

This experiment indicates that the medium is not specifically exhausted of the substances necessary for growth, nor is there a formation of specific inhibiting bodies. This has been confirmed by growing two other strains of different types of pneumococcus in dextrose broth. After a week the cultures became sterile, the organisms were removed by centrifugation, the supernatant fluids were adjusted to

a pH 7.7, and portions of each fluid were reinoculated from fresh cultures of both organisms. Growth occurred in all portions (Table X).

TABLE X.

Dextrose Broth in Which Pneumococcus Has Grown Shows No Specific Exhaustion of Fermentable Substances and No Specific Inhibiting Substances.

1 per cent dextrose broth, pH 7.7, after 7 days incubation, broth sterile, pH 5.1. The supernatant fluid after centrifugation was adjusted with sodium hydroxide to pH 7.7, reinoculated, and incubated for 48 hours.

Supernatant fluid of broth cultures of pneumococcus.	Adjusted hydrogen ion concentration of supernatant fluid.	Reinoculation of supernatant fluid with pneumococcus.	
		Type I.	Type II.
	pH	pH	pH
Type I	7.7	5.1	5.1
" II	7.7	5.1	5.1

Exhaustion of Fermentable Substances in Plain Broth.—Since growth cannot be started in either plain broth or in dextrose broth if the hydrogen ion concentration is appreciably greater than pH 7.0 to 6.8, it is evident that this degree of acidity is unfavorable for the initiation of growth. However, pneumococcus in dextrose broth of pH above 7.0 grows to a pH of 5.0, while in plain broth of the same initial pH, growth ceases at about 7.0. Is this cessation of growth a result of the attaining of that reaction, or is it due to exhaustion of fermentable substances? To test this, plain broth cultures of pneumococcus which had developed maximum acidity, were filtered through a Berkefeld filter and the filtrate was divided into several portions and reinoculated with pneumococcus. As seen in Table XI the filtrate, both at pH 7.0 and when readjusted to its initial pH 7.5, shows no growth; on the other hand, the addition of dextrose, whether to the unadjusted filtrate, or to filtrate the reaction of which has been restored to the initial pH, is sufficient to cause abundant growth. It is evident then that the cessation of growth in plain broth when the culture has reached about pH 7.0 is due, partially at least, to exhaustion of fermentable substances. The acid produced in itself does not inhibit growth. This conclusion is emphasized by Table I in which it is shown that 0.1 per cent dextrose broth reaches a pH 6.5, 0.2 per cent dextrose broth a pH 6.0, and 0.4 per cent dextrose broth a pH 5.0.

TABLE XI.

Exhaustion of Fermentable Substances in Plain Broth by Pneumococcus.

100 cc. of plain broth, initial pH 7.5, were inoculated with Type I (Strain D 46), at 37°C. for 24 hours, and filtered through a Berkefeld filter. The filtrate, pH 7.0, was reinoculated and incubated 48 hours.

Filtrate of plain broth culture of <i>Pneumococcus</i> Type I.	Hydrogen ion concentration of filtrate.	Filtrate reinoculated with pneumococcus.			
		Type I.		Type II.	
		Growth.	Hydrogen ion concentration.	Growth.	Hydrogen ion concentration.
	pH		pH		pH
Unchanged.....	7.0	—	7.0	—	7.0
Dextrose added to 1 per cent.....	7.0	+++	5.0	+++	5.1
Adjusted with NaOH.....	7.5	—	7.5	—	7.5
“ “ NaOH + 1 per cent dextrose..	7.5	+++	5.0	+++	5.0

DISCUSSION.

In this paper are presented facts thus far acquired in a study of acid production by pneumococcus when grown in the presence of fermentable substances. The strains of pneumococcus chosen are representative of the different serological types and in the majority of instances have been isolated from patients having lobar pneumonia. Some of these strains were freshly isolated, others have been under cultivation on artificial media for years. During this time their virulence has been maintained by animal passage and they have lost none of their biologic specificity. It is evident also from the protocols given that these conditions of preservation and animal passage have not affected the biochemical functions concerned in acid production. Moreover, it is apparent, for the limited number of strains studied at least, that no difference in the degree of acidity produced by the specific types of pneumococcus could be determined in media containing the test substances. In measuring the acidity produced by growth of pneumococcus use has been made of the colorimetric method for determining the hydrogen ion concentration. The limit of acid tolerance of pneumococcus in sugar-containing media, representing the final hydrogen ion concentration, is remarkably constant for all the strains studied.

The point of maximum acidity at which growth ceases has been defined as the final hydrogen ion concentration and has been found for all types of pneumococcus in carbohydrate media to be about pH 5. This final reaction is affected by the concentration of fermentable sugar in the medium up to 0.4 per cent. In the presence of this amount of dextrose, for instance, sufficient acid is produced during growth to bring the medium to the final hydrogen ion concentration of pH 5, but excess of sugar up to at least 4 per cent has no further influence on the final reaction. The rate of acid production, while a function of the size of the inoculum and of the optimum reaction of the medium, is, as previously shown by Chesney (9) and Cullen and Chesney (10), after the period of initial lag, rapid and constant until the final hydrogen ion concentration is reached.

In media containing 1 per cent maltose, saccharose, lactose, galactose, raffinose, dextrose, or inulin, strains of pneumococcus representing the four specific types produced acid to a final hydrogen ion concentration of pH 5. These results are in agreement with those of a previous study of 48 strains in which it was observed that these test substances are fermented by pneumococcus regardless of type differences.

Dernby and Avery have previously shown that the optimum hydrogen ion concentration for growth of pneumococcus in plain broth is pH 7.8. In the course of the present experiments these results have been confirmed and the observations extended to determine the optimum reaction for growth in media containing sugar. The initial reaction of all the media tested shows the optimum to be pH 7.8. The limits of hydrogen ion concentration within which growth can be initiated, however, were found to cover a somewhat wider range in carbohydrate-containing media (pH 8.3 to 6.8) than in similar media to which these substances are not added (pH 8.1 to 7.0). However, it is apparently impossible to initiate growth in a medium containing sugar if the initial reaction is more acid than pH 6.8, even though the organisms used for seeding are transplanted immediately from an actively growing culture at ranges of acidity varying from pH 7.1 to 5.

It has been found that in bacteria-free filtrates of plain broth cultures of pneumococcus, even when the filtrate is adjusted to the opti-

imum reaction, pneumococcus cannot be made to grow again unless small amounts of sugar (dextrose) are added. On the other hand, filtrates of dextrose broth cultures of pneumococcus, under the optimum conditions of reaction, apparently contained sufficient unutilized sugar to allow growth to occur on subsequent reinoculation. The ability of pneumococcus to grow, then, when reinoculated into the filtrate of a broth culture of pneumococcus of the same or different type, after the reaction of the filtrate has been readjusted to the optimum hydrogen ion concentration, appears to be dependent in part at least upon the presence of a residuum of fermentable substance left unmetabolized by previous growth. It is probable, however, that the exhaustion of fermentable carbohydrate from culture media is only one of many factors involved in the complex phenomenon of growth inhibition.

SUMMARY.

1. The optimum hydrogen ion concentration for growth of pneumococcus is pH 7.8.

2. In broth cultures growth of pneumococcus continues until a final hydrogen ion concentration of about pH 5.0 is reached, if sufficient fermentable carbohydrate (above 0.4 per cent) is present. Apparently this acidity is sufficient in itself to stop growth.

3. If less carbohydrate is present in the medium growth ceases at a lower hydrogen ion concentration, apparently because of exhaustion of carbohydrate. If no carbohydrate is present save that extracted from the meat of which the broth is made (plain broth medium), growth initiated at pH 7.8 (optimum reaction) ceases at about pH 7.0.

4. If bacteria-free filtrates of plain broth cultures in which growth has ceased are readjusted to pH 7.8 and reinoculated with pneumococcus, no growth occurs unless carbohydrate is added. However, if bacteria-free filtrates of dextrose broth cultures in which growth has ceased (pH 5) are readjusted to pH 7.8 and reinoculated with pneumococcus growth occurs.

5. Cultures of pneumococcus with all the carbohydrates which were fermentable under the conditions used, namely maltose, saccharose, lactose, galactose, raffinose, dextrose, and inulin, gave identical results in the rate of reaction change, and final hydrogen ion concentration (pH 5.0) attained.

6. The different immunological types of pneumococcus, for the limited number of strains studied, behaved alike in fermenting the carbohydrates mentioned above.

BIBLIOGRAPHY.

1. Clark, W. M., *J. Biol. Chem.*, 1915, xxii, 87.
2. Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1917, xxx, 209.
3. Ayers, S. H., *J. Bacteriol.*, 1916, i, 84.
4. Ayers, S. H., Johnson, W. T., Jr., and Davis, B. J., *J. Infect. Dis.*, 1918, xxiii, 290.
5. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.
6. Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917.
7. Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 109, 191.
8. Dernby, K. G., and Avery, O. T., *J. Exp. Med.*, 1918, xxviii, 345.
9. Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.
10. Cullen, G. E., and Chesney, A. M., *J. Exp. Med.*, 1918, xxviii, 289.

THE RELATION OF PROTEOLYTIC ENZYMES IN THE
PNEUMONIC LUNG TO HYDROGEN ION
CONCENTRATION. AN EXPLANATION
OF RESOLUTION.

By FREDERICK T. LORD, M.D.

(From the Research Laboratory of the Massachusetts General Hospital, Boston.)

(Received for publication, June 13, 1919.)

The relation of the pneumococcus to the production of acid in culture media and the acid reaction of the pneumonic lung¹ suggest that crisis and recovery may be due to local biochemical changes in the course of which the acid death-point of the pneumococcus is reached. In this paper evidence is presented of certain factors influencing resolution in pneumonia. Dernby's work on the autolysis of animal tissue² suggested methods applicable to the investigation.

Three fatal cases (Cases 1, 2, and 3) of pneumococcus pneumonia in the stage of gray or gray-red hepatization furnished the material. The involved lung was passed through a nut butter cutter, the resulting fluid from the mash filtered through cheese-cloth, and the filtrate centrifuged. The grayish sediment was washed in normal saline solution and recentrifuged. The material thus obtained is spoken of below as cellular material. Microscopic examination showed numerous pus cells, many large mononuclear cells, and numerous pneumococci. Chloroform was added to this material to kill the bacteria. Cultures made after the addition of chloroform were sterile. For all the experiments the cellular material was diluted. The diluted mixture is called cellular suspension. This cellular suspension used as enzyme proved much more active than the clear fluid which separates on standing, suggesting that proteolytic action is due to a ferment liberated by the cells.

A special object of the investigation was to determine the influence

¹ Lord, F. T., *Tr. Am. Soc. Clin. Investigation*, 1916, 8; *J. Am. Med. Assn.*, 1916, lxxvii, 1981; 1919, lxxii, 1364.

² Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.

of varying hydrogen ion concentration on the proteolytic activity of the cellular material.

Erosion of Blood Serum.—A proteolytic enzyme eroding the surface of Löffler's blood serum was readily demonstrated in the cellular material obtained from the pneumonic exudate in all three cases. The addition of chloroform to the cellular material excluded the action of living bacteria. The erosion of the surface of blood serum by the action of cellular material from various sources including the pneumonic exudate is a frequent observation in the laboratory. The relation of the proteolysis to varying degrees of acidity of the medium is an important matter.

Experiment 1.—Tubes of slanted blood serum at varying hydrogen ion concentrations were made as indicated in Table I.

TABLE I.

Method of Preparing Slanted Löffler's Blood Serum at Varying Hydrogen Ion Concentrations for Testing Proteolytic Action.

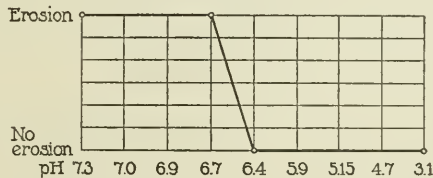
Blood serum 750 cc., dextrose bouillon 250 cc., glycerol 15 cc. To 30 cc. of this mixture was added the indicated amount of N hydrochloric acid or 0.1 N sodium hydroxide + water to 36 cc.

Flask No.	N HCl	0.1 N NaOH	Water.	pH
	cc.	cc.	cc.	
a	} Slanted blood serum immersed in solutions of known hydrogen ion concentration.*			3.1
b				4.7
1	2.0		4.0	5.15
2	0.9		5.1	5.9
3	0.7		5.3	6.4
4	0.5		5.5	6.7
5		2.0	4.0	7.0
6		4.5	1.5	7.3

* The creation of a higher acidity than 5.1 by the addition of acid to the serum prevented the inspissation of the medium and slants of coagulated blood serum already made were allowed to stand covered with standard solutions containing 0.2 M acid potassium phthalate or 0.2 M potassium dihydrogen phosphate and 0.2 M sodium hydroxide of hydrogen ion concentration varying from 4.6 to 2.2. Samples of the fluid were removed at intervals until the hydrogen ion concentration had become constant. By this means, after 3 days slants were obtained of Löffler's blood serum, the fluid covering which had a pH of 3.1 to 5.15.

The water of condensation was removed before the experiment. Cellular material obtained from Case 3 was used. The cellular suspension was made by suspending four drops of cellular material in ten drops of standard solutions of known hydrogen ion concentration and the hydrogen ion concentration of the resulting cellular suspension was then determined. To the surface of the slanted blood serum³ of varying hydrogen ion concentrations a cellular suspension of nearly the same hydrogen ion concentration was added. After incubation erosion of the surface of the medium was observed on the slants with a pH of 7.3 to 6.7 (Text-fig. 1).

Other experiments confirmed these results and showed that the precaution of controlling the hydrogen ion concentration of the cellular suspension used as enzyme was unnecessary. The enzyme proved active at and below 6.7 and inactive at and above 6.4; *i.e.*,



TEXT-FIG. 1. Proteolytic enzyme eroding the surface of Löffler's blood serum. To slanted blood serum with water of condensation of hydrogen ion concentrations varying from 7.3 to 3.1 cellular material suspended in solutions of approximately the same hydrogen ion concentration was added. Erosion of the medium was noted in the slants with a pH of 7.3 to 6.7. No erosion occurred at a pH of 6.4 or higher.

on the more acid end of the scale. Chloroform was added to the cellular material and no growth of bacteria was observed on the surface of the medium.

Gelatin Method.—A stock solution was made by dissolving 350 gm. of gelatin in 625 cc. of hot water and the solution passed through cheese-cloth. 1 gm. of thymol suspended in water was added and the solution made up to 1 liter with water.

Before the experiments 200 cc. of this stock solution were taken and diluted to 500 cc. To 15 cc. of this solution hydrochloric acid or sodium hydroxide was added to vary the hydrogen ion concentrations and the volume was made up to 30 cc. with distilled water. Two series of gelatin solutions with hydrogen ion concentrations

³ Kept horizontal after the addition of the cellular suspension.

varying from 8.0 to 2.0 were thus prepared, one to serve as control and the other for the experiment.

Experiment 2.—To each flask in one of the two series three drops of the cellular suspension obtained from the pneumonic lung (Case 1) and used as enzyme were added. The cellular suspension was made from 0.5 cc. of cellular material (to which chloroform had been added) diluted with 10 cc. of sterile distilled water. Cultures from the cellular material showed no growth. Both control and experimental solutions were placed in the incubator. After $4\frac{1}{2}$ and 22 hours 5 cc. samples were removed from each of the flasks in the two series, placed in thin test-tubes, and cooled for 15 minutes in ice water. On removal from the ice bath the degree of liquefaction of the gelatin in the two series was compared. For the purpose of recording the result the tubes found completely solid were classed as 0 and those completely liquid as 6, intervening numbers indicating a corresponding degree of liquefaction. Cultures from the experimental solutions at the termination of the experiment showed no growth.

Experiment 3.—An exactly similar experiment was performed with cellular material obtained from the pneumonic lung in Case 2.

The presence of an enzyme acting at both acid and alkaline ends of the scale was suggested by some degree of liquefaction of the gelatin in the experimental flasks, but the curve showed a tendency to rise progressively toward the ends of the scale, making the interpretation doubtful. The experiments with gelatin lose in value on account of an almost exactly similar behavior of the controls. Loeb⁴ has shown that gelatin at its isoelectric point ($C_H = 2.10^{-5}$) is practically insoluble, but when it is transformed into a salt by the addition of an acid (or a base) it becomes soluble provided it is in combination with a monovalent ion. Although the gelatin can be brought back to the critical hydrogen ion concentration by adding a calculated amount of acid or alkali before the samples are placed in the ice bath, this procedure further complicates the experiment and does not seem to offer more advantage than the use of the controls. The presence of an enzyme liquefying gelatin could not be demonstrated by this method.

Peptone Method.—Seven solutions of Witte's peptone with hydrogen ion concentrations varying from a pH of about 8.5 to 4.0 were prepared according to the method indicated in Table II. To por-

⁴ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 363.

tions removed from each of the seven flasks equal amounts of a homogeneous cellular suspension used as enzyme were added. The amino-acid nitrogen in the solutions was determined by Van Slyke's method⁵ with the micro-apparatus devised by him.⁶ The cells were first removed by centrifuging the solutions. The total nitrogen determinations were done in duplicate by Folin and Denis' method,⁷ by direct Nesslerization, or by the Kjeldahl method.

TABLE II.

Method of Preparing Peptone at Varying Hydrogen Ion Concentrations for Testing Proteolytic Action.

20 cc. of 4 per cent peptone (Witte's) + the indicated amount of 0.5 M phosphates + hydrochloric acid or sodium hydroxide + distilled water to 90 cc.

Flask No.	Chloroform.	0.1 N HCl	0.1 N NaOH	0.5 M phosphates.		pH indicated.	pH determined.
				KH ₂ PO ₄	Na ₂ HPO ₄		
	cc.	cc.	cc.	cc.	cc.		
1	1	16.0		10.0	0	4.0	4.0
2	1	8.0		9.0	1.0	5.2	5.2
3	1	3.0		5.0	5.0	6.8	6.6
4	1			2.0	8.0	7.3	7.3
5	1		3.0	1.0	9.0	7.7	7.8
6	1		8.0	0.5	9.5	8.0	8.0
7	1		14.0	0	10.0	8.5	8.5

The composition of the solutions used for the determination of peptone-splitting enzyme in the pneumonic lung was the same as that used by Dernby in his study of autolysis of animal tissue with the exception of a change in the amount of peptone from 40 cc. to 20 cc. and the water from 180 cc. to 90 cc.

Experiment 4.—To 0.5 cc. of cellular material obtained from a Pneumococcus Type II pneumonia (Case 1) chloroform was added and the material was allowed to stand over night to plasmolyze. Cultures made after the addition of the chloroform were sterile. A cellular suspension was made by adding to the 0.5 cc. 16 cc. of distilled water. 28 cc. from each of the seven flasks (Table II) were removed to separate flasks and to each were added 2 cc. of the homogeneous cellular suspension used as enzyme and four drops of chloroform. The remainder

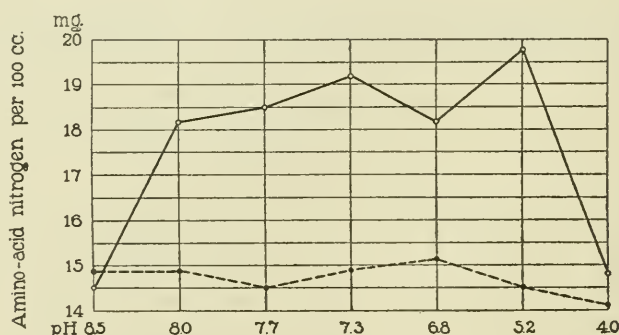
⁵ Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

⁶ Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxiii, 407.

⁷ Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

of the original peptone solutions to serve as controls and the experimental solutions were allowed to remain in the incubator for 44 hours. The results of the determination of the amino-acid nitrogen are indicated in Table III and graphically recorded in Text-fig. 2.

As indicated in Table III the control solutions already contained from 14.3 to 15.1 mg. of amino-acid nitrogen per 100 cc., the slight variations in the different flasks indicating the experimental error which does not exceed 0.8 mg. per 100 cc. Though equal amounts of homogeneous cellular suspension were added to the experimental flasks the resulting increase in the amino-acid nitrogen varies at the different hydrogen ion concentrations and reaches a maximum in the



TEXT-FIG. 2. Peptone-splitting enzyme in the pneumonic lung from Case 1. The action of cellular material obtained from pneumonic lung in splitting Witte's peptone to amino-acid nitrogen after incubation for 44 hours is shown. The solid line indicates solutions with cellular material, the broken line the same solutions without cellular material (control).

flask with a hydrogen ion concentration of 5.2 pH, falling sharply from this point toward the more acid side and slowly toward the more alkaline side until a pH of 8.0 is reached when the curve again falls abruptly. The lack of variation beyond the experimental error in the control flasks under the conditions of the experiment and the much wider variations at the different hydrogen ion concentrations in the experimental flasks suggest that an enzyme of optimum activity at a pH of 5.2 is the cause of the increased liberation of amino-acid nitrogen. The experiment shows that the amount of amino-acid nitrogen in the peptone solutions without cellular suspension does not

TABLE III.

Peptone-Splitting Enzyme in the Pneumonic Lung from Case 1.

Flask No.	Initial pH.	Control. Amino-acid nitrogen per 100 cc.	Experiment. Amino-acid nitrogen per 100 cc.	Cultures from flasks.	
				Before experiment.	At end of experiment.
		mg.	mg.		
1	4.0	14.3	14.7	No growth.	No growth.
2	5.2	14.5	19.9	" "	" "
3	6.8	15.1	18.2	" "	5 white colonies.
4	7.3	14.7	19.1	" "	No growth.
5	7.7	14.5	18.5	" "	" "
6	8.0	14.7	18.2	" "	" "
7	8.5	14.7	14.5	" "	" "

Control solution, total nitrogen (Folin and Denis method) 90 mg. per 100 cc. The five colonies obtained from the transplant from No. 3 were a contaminating organism, not pneumococci.

appreciably change. The experiment is open to the criticism that amino-acid nitrogen preformed in the cellular suspension may have been added to the peptone solutions, but other experiments have shown that there is too small an amount of preformed amino-acid nitrogen in the $\frac{1}{16}$ cc. of cellular material added to each flask to influence the individual readings. Any possible error from this source is eliminated in the following experiments.

Experiment 5.—An experiment was performed using cellular material obtained from another fatal case (Case 3) of *Pneumococcus* Type I pneumonia. The cellular material treated as before with chloroform was sterile. The cellular suspension was made by diluting 0.5 cc. of cellular material with 16 cc. of distilled water. 2 cc. of the cellular suspension were added to 28 cc. from each of seven freshly prepared solutions of Witte's peptone of the composition indicated in Table II. 0.3 cc. of chloroform was added to each flask.

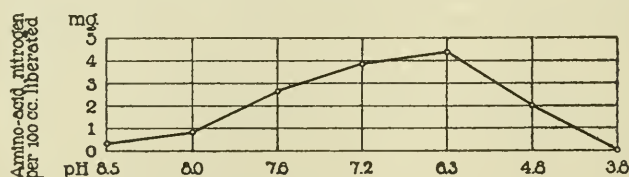
The amount of amino-acid nitrogen in each flask averaged 14.3 mg. per 100 cc. before the addition of the cellular material. The total nitrogen (Kjeldahl method) in the peptone solutions averaged 103.5 mg. per 100 cc. before and 106 mg. after the addition of the cellular suspension. The cellular suspension therefore added about 2.5 mg. of total nitrogen per 100 cc. This amount of total nitrogen in the cellular suspension is too small to account for more than a fraction of the amino-acid nitrogen liberated.

The amount of amino-acid nitrogen in each flask was determined before and after incubation for 42 hours. The results are indicated in Table IV. The increase in amino-acid nitrogen liberated is graphically shown in Text-fig. 3. The hydrogen ion concentration of the flasks changes very little during the experiment. Cultures from the flasks during the experiment were sterile. A peptone-splitting enzyme acting most strongly at a hydrogen ion concentration of 6.3 is indicated.

TABLE IV.

Peptone-Splitting Enzyme in the Pneumonic Lung from Case 3.

Flask No.	Chloroform.	Initial pH.	Final pH.	Amino-acid nitrogen per 100 cc.		Cultures during experiment.
				Before incubation.	After incubation.	
	cc.			mg.	mg.	
1	0.3	4.0	3.8	14.9	14.9	No growth.
2	0.3	4.9	4.8	15.6	17.6	" "
3	0.3	6.5	6.3	15.0	19.4	" "
4	0.3	7.1	7.2	14.6	18.5	" "
5	0.3	7.6	7.6	15.5	18.4	" "
6	0.3	8.0	8.0	15.2	16.5	" "
7	0.3	8.5	8.5	14.5	15.1	" "



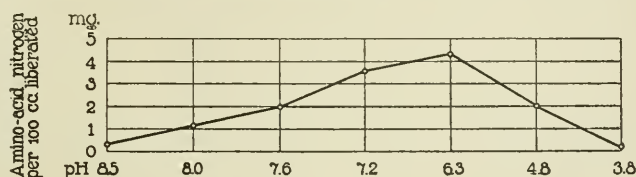
TEXT-FIG. 3. Peptone-splitting enzyme in the pneumonic lung from Case 3. The increase in amino-acid nitrogen at varying hydrogen ion concentrations in flasks containing Witte's peptone and cellular material from the pneumonic lung after incubation for 42 hours is shown.

Experiment 6.—0.5 cc. of cellular material similarly obtained from another fatal case of *Pneumococcus* Type I pneumonia (Case 2) and treated as before with chloroform was diluted with 16 cc. of distilled water. Cultures from the cellular material after the addition of chloroform were sterile. 2 cc. of the cellular suspension were added to 28 cc. from each of the seven original solutions of Witte's peptone used in Experiment 5. The amino-acid nitrogen in the peptone solutions was already 14.3 mg. per 100 cc.; the total nitrogen (Kjeldahl method) averaged 103.5 mg. per 100 cc. before and 105 mg. per 100 cc. after the addition of the cel-

lular suspension, indicating that the cellular suspension added about 1.5 mg. of total nitrogen per 100 cc. This amount of total nitrogen in the cellular suspension is too small to account for more than a small fraction of the amino-acid nitrogen liberated from the Witte's peptone. The amount of amino-acid nitrogen was determined in each flask before and after incubation for 42 hours, with the result recorded in Table V and graphically presented in Text-fig. 4. As in the preceding experiment a peptone-splitting enzyme acting most strongly at a hydrogen ion concentration of 6.3 is indicated. Cultures from the flasks before, during, and after the experiment were sterile.

TABLE V.
Peptone-Splitting Enzyme in the Pneumonic Lung from Case 2.

Flask No.	Chloroform.	Initial pH.	Amino-acid nitrogen per 100 cc.		Cultures before, during, and after experiment.
			Before incubation.	After incubation	
	cc.		mg.	mg.	
1	0.3	3.8	14.8	15.0	No growth.
2	0.3	4.8	14.7	16.7	" "
3	0.3	6.3	14.5	18.8	" "
4	0.3	7.2	14.8	18.2	" "
5	0.3	7.6	15.0	17.0	" "
6	0.3	8.0	15.0	16.1	" "
7	0.3	8.5	14.5	14.8	" "



TEXT-FIG. 4. Peptone-splitting enzyme in the pneumonic lung from Case 2. The increase in amino-acid nitrogen at varying hydrogen ion concentrations in flasks containing Witte's peptone and cellular material from the pneumonic lung after incubation for 42 hours is shown.

SUMMARY.

Evidence is given of the presence in the cellular material obtained from the pneumonic lung of a proteolytic enzyme digesting coagulated blood serum at hydrogen ion concentrations of 7.3 to 6.7 and inactive at higher; *i.e.*, more acid concentrations.

In addition, evidence is brought forward of the presence in the cellular material from the pneumonic lung of a proteolytic enzyme splitting peptone to amino-acid nitrogen. This enzyme is operative at hydrogen ion concentrations from 8.0 to 4.8, but most active at 6.3 or 5.2.

These findings may be regarded as having a bearing on resolution in pneumonia. During the course of the disease a gradual increase in the hydrogen ion concentration of the exudate probably takes place. With the breaking down of cellular material an enzyme digesting protein (fibrin) in weakly alkaline and weakly acid media may be liberated. With a gradual increase in the hydrogen ion concentration of the pneumonic lung the action of this enzyme probably ceases. An enzyme capable of splitting peptone to amino-acid nitrogen is probably active during the proteolysis of the fibrin and further activated when the hydrogen ion concentration of the pneumonic lung is increased to within its range of optimum activity at a pH of 6.3 and 5.2. By this means it may be conceived that the exudate is dissolved and resolution takes place.

I am indebted to Dr. R. N. Nye for assistance, to Miss A. S. Minot for the chemical analyses, and to Miss E. W. Bicknell and Miss Margaret Herrick for technical aid.

THE RELATION OF THE PNEUMOCOCCUS TO HYDROGEN ION CONCENTRATION, ACID DEATH-POINT, AND DISSOLUTION OF THE ORGANISM.*

BY FREDERICK T. LORD, M.D., AND ROBERT N. NYE, M.D.

(From the Research Laboratory of the Massachusetts General Hospital, Boston.)

(Received for publication, June 23, 1919.)

Lord¹ has called attention to the probable importance of an increase in the hydrogen ion concentration in the pneumonic lung in inhibiting the growth of the pneumococcus and favoring enzymatic action. In this article we wish to elaborate certain aspects of the relation of the pneumococcus to changes in acidity. The hydrogen ion concentrations were determined by the colorimetric method with standard solutions made according to Clark and Lubs' directions.² As indicators, phenolsulfonephthalein was used on hydrogen ion concentrations ranging from 8.0 to 6.2, and sodium alizarin sulfonate from 6.0 to 4.0. Colored solutions were either dialyzed or the determinations made by the comparator rack method.

Relation of the Pneumococcus to Varying Hydrogen Ion Concentrations.

Experiment 1.—A 1 per cent glucose bouillon culture of *Pneumococcus* Type II with an initial hydrogen ion concentration of 7.65 shows an increasing multiplication of the organism, as indicated in Text-fig. 1, and reaches a maximum of growth in about 12 hours. The acidity increases to a pH of 5.25 at which there is a rapid fall in the number of living pneumococci. With the increase in the acidity to a pH of 5.15 no living organisms remain in the flask.

Reinoculation of such a flask in which the pneumococcus has grown and died out and the removal of samples at intervals show that living

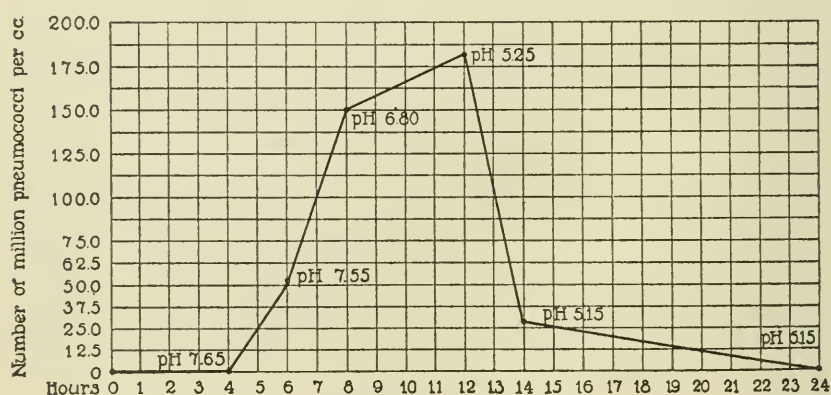
* Presented at the meeting of the American Society for Clinical Investigation, Atlantic City, N. J., June 14, 1919.

¹ Lord, F. T., *Tr. Am. Soc. Clin. Investigation*, 1916, 8; *J. Am. Med. Assn.*, 1916, lxxvii, 1981; 1919, lxxii, 1364.

² Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.

organisms can be obtained from the flask for an interval of 1 hour and that no growth is obtained on the transplants taken on and after 3 hours. This experiment suggests that a hydrogen ion concentration of about 5.15 may be regarded as the degree of acidity which will almost immediately kill the organism, but the suggestion is open to the objection that other factors than the acidity may be present and exert a bactericidal action.

Other strains of pneumococci of Types I, II, and III, allowed to grow in 1 per cent glucose bouillon until the culture becomes sterile,



TEXT-FIG. 1. The acid death-point of *Pneumococci* Type II. The results with 1 per cent glucose bouillon are shown.

produce a final acidity of about the same hydrogen ion concentration. No noteworthy difference among the three fixed types has been noted, with the following exception. One strain of Type I pneumococcus obtained from a fatal case of pneumonia remained alive for at least 10 days after the culture had reached a hydrogen ion concentration of 4.5.

Experiment 2.—Repeated reinoculation of the flask (Experiment 1) after the addition of a sufficient amount of sodium hydroxide to neutralize the titrable acidity results in growth for progressively longer intervals as shown in Table I. Partial exhaustion of the material from which the acid is formed may be regarded as the explanation of the increased viability of the culture.

The experiment suggests that the acidity is the most important factor in the death of the organism since without other change in the conditions of the experiment than the neutralization of the acidity and the lapse of time, the pneumococcus will repeatedly grow in the same flask and for progressively longer periods. However, some other factor than the acidity may also play a part in the death of the organism.

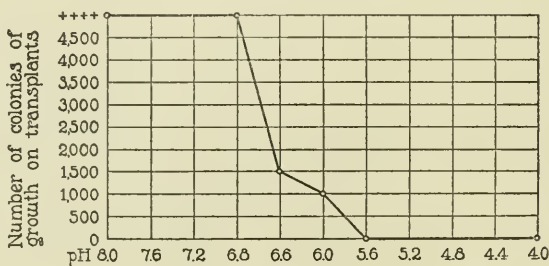
TABLE I.
Growth of Pneumococcus in Realkalinized Glucose Bouillon.

Inoculation.	pH	N NaOH required to neutralize 5 cc.	Duration of growth.
		cc.	
1st.....	7.65-5.15	0.22	14-24 hrs.
2nd.....	-5.9	0.17	4 days.
3rd.....	-6.9	0.115	6 "
4th.....	-7.0		22+ "

1 per cent glucose bouillon inoculated with Type II pneumococcus allowed to die out and repeatedly realkalinized and reinoculated.

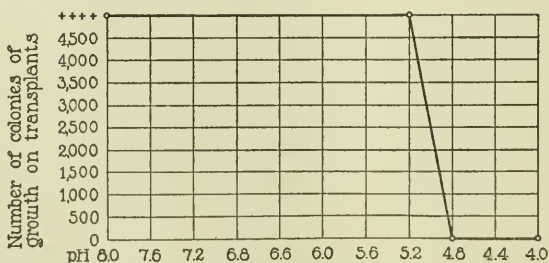
Experiment 3.—In order to test the relation of the pneumococcus to the acidity of the media and to exclude as far as possible the presence of inhibiting substances arising as a result of the growth of the organism, suspensions of washed living pneumococci were made in standard solutions of known hydrogen ion concentrations as follows: Ten drops of each of Clark and Lubs' solutions were placed in small tubes and sterilized in the Arnold sterilizer. Ten drops of a suspension in normal saline solution of the bacterial sediment washed with normal saline solution from an actively growing culture of the pneumococcus were added to each of the solutions of varying hydrogen ion concentrations. The culture of the pneumococcus (Type II) was at the height of its growth and there was no evidence of agglutination or sedimentation in the flask. The addition of the bacteria made a slightly cloudy suspension. The hydrogen ion concentration was not changed by the procedure. The tubes were placed in the incubator and transplants made to the surface of blood serum after 5½ hours, by smearing one loop of the material on the surface, with the result indicated in Text-fig. 2. No growth of organisms was obtained from the tubes at 5.6 or higher hydrogen ion concentrations. An abundant growth of colonies, too numerous to count, was obtained from the tubes at 6.8 and lower hydrogen ion concentrations. About 1,000 colonies were obtained from the tube with a pH of 6.0 and 1,500 from that with a pH of 6.6.

The experiment suggests that irrespective of any bactericidal substances which may be formed in culture media in consequence of the bacterial growth, the pneumococcus will not live for $5\frac{1}{2}$ hours in the presence of hydrogen ion concentrations from 5.6 to 4.0 under the conditions of the experiment, and that some inhibition is present in concentrations from 5.6 to 6.8, beyond which, toward the alkaline end of the scale, more living organisms are present.



TEXT-FIG. 2. The acid death-point of *Pneumococci* Type II. Suspensions were made in normal saline solution of washed pneumococci at different hydrogen ion concentrations. Transplants were made from these solutions after $5\frac{1}{2}$ hours in the incubator.

Experiment 4.—In Text-fig. 3 the result of a similar experiment with Type III pneumococcus is graphically presented. Bactericidal action of hydrogen ion concentrations from 4.8 to 4.0 acting for 1 hour is indicated.



TEXT-FIG. 3. The acid death-point of *Pneumococci* Type III. Suspensions were made in normal saline solution of washed pneumococci at different hydrogen ion concentrations. Transplants were made from these solutions after 1 hour in the incubator.

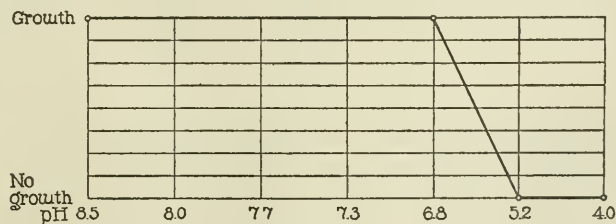
Experiment 5.—To avoid the possibility that the particular solutions used in these experiments (Nos. 3 and 4) may have influenced the result, the experiment was repeated using peptone solutions,³ of the composition indicated in Table II, of hydrogen ion concentrations varying from 8.5 to 4.0. A large amount of suspension of washed pneumococci (Type II) was added to small amounts of these solutions. After 19 hours in the incubator growth was obtained in transplants from the solutions with a pH of 8.5 to 6.8 as shown in Text-fig. 4. No growth was

TABLE II.

Composition of Solutions for the Determination of the Acid Death-Point.

20 cc. of 4 per cent peptone (Witte's) + hydrochloric acid or sodium hydroxide + 0.5 M phosphates + water to 90 cc.

Flask No.	0.1 N HCl	0.1 N NaOH	0.5 M phosphates.		pH
			KH ₂ PO ₄	Na ₂ HPO ₄	
	cc.	cc.	cc.	cc.	
1	16.0		10.0		4.0
2	8.0		9.0	1.0	5.2
3	3.0		5.0	5.0	6.8
4			2.0	8.0	7.3
5		3.0	1.0	9.0	7.7
6		8.0	0.5	9.5	8.0
7		14.0		10.0	8.5



TEXT-FIG. 4. The acid death-point of *Pneumococci* Type II. 7.5 cc. of suspensions of washed pneumococci in normal saline solution were added to 2.5 cc. of peptone solutions at hydrogen ion concentrations from 8.5 to 4.0 obtained by adding varying amounts of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide and 0.5 M phosphates. Incubation was for 19 hours. Growth was obtained on transplants from solutions with pH 8.5 to 6.8 and no growth with pH 5.2 to 4.0.

³ The composition was suggested by Dernby's work (Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179).

obtained from those at 5.2 and 4.0. The experiment indicates that in solutions at a pH of 6.8 and a lower hydrogen ion concentration *Pneumococcus* Type II will live for 19 hours.

While it is apparent from these experiments that the acidity of the medium has an important bearing on the death of the pneumococcus, it is desirable to obtain data on the relation of the time of exposure to the death of the organism.

Experiment 6.—For this purpose weak solutions of bouillon at varying hydrogen ion concentrations were prepared according to the method indicated in Table III. 5 cc. from each flask were removed to sterile test-tubes. The tubes were

TABLE III.

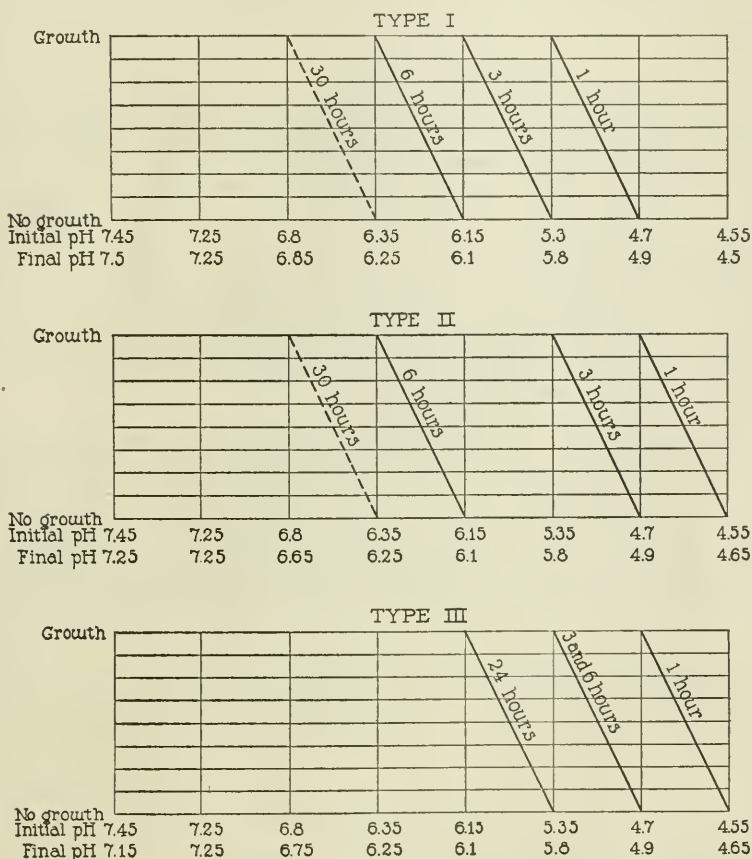
Composition of Solutions for the Determination of the Acid Death-Point.

20 cc. of plain bouillon, hydrochloric acid, and 0.5 M phosphates + water to 90 cc.

Flask No.	0.1 N HCl	0.5 M phosphates.		pH
		KH ₂ PO ₄	Na ₂ HPO ₄	
	cc.	cc.	cc.	
1	12.0	10.0	0.0	4.55
2	10.0	9.5	0.5	4.7
3	8.0	9.0	1.0	5.35
4	6.0	7.5	2.5	6.15
5	4.0	6.5	3.5	6.35
6	2.0	4.0	6.0	6.8
7	0	2.0	8.0	7.25
8	0	0.0	0.0	7.45

set up in triplicate and one drop of a suspension in salt solution of washed pneumococci of Type I, II, or III was added to each tube of the three series. The tubes were then placed in the incubator and transplants made on blood serum at intervals of 1, 3, and 6 hours. It is apparent from the graphic presentation of the results in Text-fig. 5 that there is a time element in the bactericidal action of acidity. Thus Type I was killed by exposure to a hydrogen ion concentration of 4.5 and 4.7 for 1 hour, but survived an acidity of 5.3 for this interval only to succumb to it after exposure for 3 hours. A pH of 6.15 was withstood for 3 but not for 6 hours and living organisms were obtained after this interval at a pH of 6.35. Type II showed slightly less susceptibility to acid in this experiment. Type III is still less susceptible. Other experiments indicate that there is no constant difference in the acid death-point of the fixed types. Determination of the hydrogen ion concentrations of the mixtures before the experiment and 24 hours later showed no noteworthy change.

From these and other experiments it may in general be said that in fresh culture media the pneumococcus withstands a pH of about 5.3 for 1 hour, of 5.6 for 3 hours, and of 6.1 for 6 hours. It is to be noted (Experiment 1) that when reinoculated into culture media in which the organism has grown and died out it survives a pH of about 5.1



TEXT-FIG. 5. The relation of time and acidity to the death of pneumococci. Suspensions of pneumococci in normal saline solution were added to bouillon solutions at hydrogen ion concentrations varying from 7.45 to 4.5. Transplants were made after varying intervals. The 30 hour periods in Types I and II are taken from another similar experiment.

for 1 hour, thus showing by comparison that there is no essential difference in the acid death-point in fresh culture media and that in culture media containing whatever products may be liberated by the growth and death of the organisms. The acidity is therefore the most important factor.

In contrast to the preceding experiments the following experiments indicate that cultures of pneumococci remain viable when the acidity is low.

Experiment 7.—1 per cent glucose calcium carbonate bouillon with an initial pH of 7.4 was inoculated with pneumococci June 10, 1915. Transplants every few days to June 28 showed growth. On June 28 the hydrogen ion concentration was 6.7. Transplants at less frequent intervals showed growth through December 15. The hydrogen ion concentration was not again determined. The total duration of life was about 6 months.

Experiment 8.—Plain bouillon with an initial pH of 7.4 was inoculated with pneumococci June 10, 1915. Transplants every few days to June 28 showed growth. The pH was then 7.4 as before. Further transplants gave growth through December 15. The hydrogen ion concentration was not again determined. The total duration of life was about 6 months.

Experiments 6 to 8 suggest that within the range of hydrogen ion concentrations investigated (pH 7.45 to 4.5) the pneumococcus is killed by hydrogen ion concentrations above about 6.8 with a rapidity which bears a direct relation to the hydrogen ion concentration, *i.e.* the greater the hydrogen ion concentration the more rapid the death, and that the pneumococcus will continue to live in suitable culture media at a pH of about 6.8 to 7.4 for at least many days.

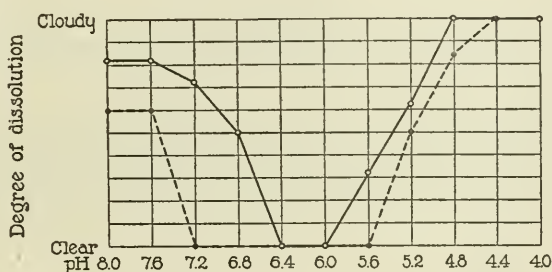
The following experiments suggest that some other factor than the acidity alone is responsible for destruction of pneumococci.

Influence of Varying Hydrogen Ion Concentrations on Dissolution of Pneumococci.

A striking phenomenon which may be spoken of as dissolution is observed when to ten drops of standard solutions of known hydrogen ion concentration ten drops of a suspension of washed pneumococci are added and the resulting slightly cloudy suspension of pneumococci is placed in the incubator. A clearing of the tubes at a hydrogen ion concentration of about 5.0 to 6.0 is observed. The tubes on the more

acid side of the scale remain homogeneously cloudy, although there may be some diminished density at 4.4 and 4.8 and there is constantly some clearing which increases with time on the more alkaline side of the scale. The clearing of the suspension at different hydrogen ion concentrations is graphically indicated in Text-figs. 6 to 8.

Microscopic examination of films from the different tubes shows that the pneumococci in the tubes at the acid end of the scale maintain their morphology, but for the most part become Gram-negative. Examination of films from the cleared suspensions within the range of a pH of about 5.0 to 6.0 shows a few poorly stained organisms, with the shadowy remains of others. The pneumococci are disintegrated and many remnants show as staining points. All are decolorized by

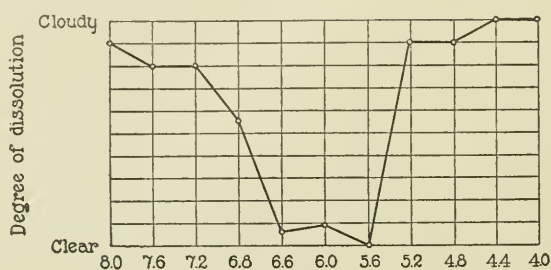


TEXT-FIG. 6. The degree of dissolution of Pneumococci Type I after 7 hours (solid line) and 24 hours (broken line). The suspensions of washed pneumococci in normal saline solution were added to solutions of different hydrogen ion concentrations.

Gram's stain. Films from the tubes at the alkaline end of the scale show some but much less disintegration. Many organisms retain the Gram stain.

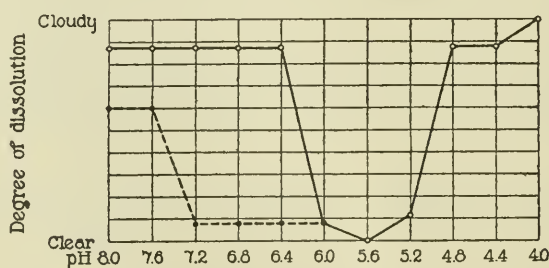
The explanation of the dissolution of the organisms is uncertain. It does not seem to be due to the particular chemical substances, sodium chloride, acid potassium phthalate, potassium dihydrogen phosphate, and sodium hydroxide used in this experiment, since the sodium hydroxide is the only variable factor and the curve of dissolution does not correspond to the curve of sodium hydroxide concentration. Moreover, the same curve of dissolution occurs in other standard solutions without sodium hydroxide. The addition of ten drops of

suspension of washed pneumococci to these solutions changes the hydrogen ion concentration little or not at all. Calculation shows that



TEXT-FIG. 7. The degree of dissolution of Pneumococci Type II after 5½ hours. The suspensions of washed pneumococci in normal saline solution were added to solutions of different hydrogen ion concentrations.

a change in the molecular concentration of the solutions is an unlikely explanation. It is not due to the acidity alone, since if it were the acid end of the scale would not remain cloudy. It may be con-
 jec-



TEXT-FIG. 8. The degree of dissolution of Pneumococci Type III after 1 hour (solid line), and 18 hours (broken and solid line). The suspensions of washed pneumococci in normal saline solution were added to solutions of different hydrogen ion concentrations.

cluded that some other factor besides the acidity is responsible and the activation of an enzyme derived from the bacteria themselves may be the explanation.

CONCLUSIONS.

1. In the growth and death of the pneumococcus in fluid media containing 1 per cent glucose the production of acid is the most important bactericidal factor.

2. 1 per cent glucose bouillon cultures of the pneumococcus allowed to grow and die out usually reach a final acidity of a pH of about 5.1.

3. At a hydrogen ion concentration of about 5.1 or higher, the pneumococcus does not survive longer than a few hours.

4. In hydrogen ion concentrations of about 6.8 to 7.4 the pneumococcus may live for at least many days.

5. In the intervening hydrogen ion concentrations, between 6.8 and 5.1, the pneumococcus is usually killed with a rapidity which bears a direct relation to the hydrogen ion concentration; *i.e.*, the greater the acidity the more rapid is the death.

6. Cloudy suspensions of washed pneumococci in hydrogen ion concentrations varying from 8.0 to 4.0 show, after incubation, dissolution of organisms in lower hydrogen ion concentrations than about 5.0. This dissolution is most marked at about 5.0 to 6.0. Some dissolution also takes place toward the more alkaline end of the scale. No dissolution occurs at the most acid end of the scale.

We are indebted to Miss E. W. Bicknell and Miss Margaret Herrick for technical assistance.

ETIOLOGY OF YELLOW FEVER.

IX. MOSQUITOES IN RELATION TO YELLOW FEVER.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 3, 1919.)

That a certain species of mosquito serves as a carrier of yellow fever has long been suspected. Finlay¹ supported this theory as early as 1881 and went so far as to advise conferring immunity upon non-immune persons by causing them to be bitten by mosquitoes (*Stegomyia calopus*) which had previously fed on a yellow fever patient. Thenceforth *Stegomyia calopus* became the center of attention in yellow fever investigations, the theory being experimentally confirmed finally by Reed, Carroll, Agramonte, and Lazear,² who successfully transmitted yellow fever to non-immune human subjects by allowing the infected stegomyias to bite them. The successful eradication of yellow fever by Gorgas³ from various endemic centers in the western hemisphere through anti-stegomyia campaigns amply attests the correctness of this important discovery. Reed and his coworkers established the fact that the virus exists in the peripheral blood during the first 3 days of the illness and that the mosquitoes, which have fed on a yellow fever patient during this period, become capable, after about 10 days, of transmitting the infection by their bite to a normal person. Hence they assumed that the virus required a period of at least 10 to 12 days of extrinsic incubation during which it must pass through a life cycle, as was known to be the case with the parasite of malaria. The necessity for an extrinsic incubation of about 12 days was pointed out by Carter⁴ in his epidemiological studies of yellow fever. This theory, coupled with the improbability of a bacterial origin of the disease, brought about the general impression that the virus was a protozoan, although nothing definitely objective was adduced in support of this view.

¹ Finlay, C., El mosquito hipotéticamente considerado como agente de trasmisión de la fiebre amarilla, *An. r. Acad. cien. méd. Habana*, 1881-82, xviii, 147.

² Reed, W., Carroll, J., Agramonte, A., and Lazear, J. W., The etiology of yellow fever, *Am. Pub. Health Assn., 28th Meeting*, 1900; also *Senate Doc. No. 822, 61st Congr., 3rd Sess.*, 1911, 56.

³ Gorgas, W. C., Sanitation in Panama, New York and London, 1915.

⁴ Carter, H. R., Note on the spread of yellow fever in houses. Extrinsic incubation, *Med. Rec.*, 1901, lix, 933.

Reference has already been made^{5,6} to the fact that the virus of yellow fever passed through the pores of a Berkefeld or a Chamberland filter. This point is important in excluding as the inciting agent of the disease any organism incapable of passing through these filters which might be found incidentally in the blood or tissues of yellow fever patients. Some persons believe that the virus is ultramicroscopic, at least with the magnification possible at the present time.

The characteristics of the organism isolated from the yellow fever cases in the present investigation conformed with all the other known characteristics of the yellow fever virus, and it became necessary to determine whether or not it behaved like the latter in relation to mosquito transmission. That it does, under certain conditions, is shown in the experiments to be described.

Material and Mode of Experiments.

Larvæ of *Stegomyia calopus* collected from houses in Guayaquil were brought to the laboratory.⁷ They varied in stage from the very young to the pupal form.

For the purpose of maintaining the imagos which emerged from these larvæ special cages were constructed consisting of a square wooden frame with wire net on all but two opposing lateral walls, which were solid pieces of wood with a round opening in the center for the insertion of the arm or a glass of water from either side of the cage. The opening was covered by a well fitting sliding door which could be drawn to one side and slipped back in place. A long glass cylinder about 8 inches long and 2 inches in diameter with one end closed was used for handling individual mosquitoes. When the specimens were to be killed for examination a piece of cotton saturated with chloroform was put at the bottom of the cylinder. For histological study the mosquitoes were placed in fixing fluid such as sub-

⁵ Reed, W., and Carroll, J., The etiology of yellow fever; a supplemental note, *Senate Doc. No. 822, 61st Congr., 3rd Sess.*, 1911, 149.

⁶ Marchoux, Salimbeni, and Simond, La fièvre jaune, *Ann. Inst. Pasteur*, 1903, xvii, 665.

⁷ These were obtained through the cooperation of the officials of the Municipal Board of Health of Guayaquil.

limate-alcohol, Zenker's fluid, or 10 per cent formaldehyde, after the wings and legs were trimmed off. Mosquitoes engorged with blood usually sink to the bottom in a short time, but those which are unfed or have already digested the blood do not sink for many hours or even days, and it was necessary to keep them within the fixing fluid by forcing them down with a piece of absorbent cotton. In some instances a minute needle puncture into the abdomen or thorax, or both, facilitated the prompt penetration of the fixative agents. The specimens fixed in sublimate-alcohol or Zenker's fluid were washed in water for 48 hours and then transferred to 50 per cent alcohol, in which they were kept until worked out later for serial sections.

The larvæ showed a surprising resistance to phenol (3 per cent), formaldehyde (10 per cent), sublimate-alcohol (two parts of saturated aqueous solution of mercuric chloride and one part of absolute alcohol), and Zenker's fluid, being capable of surviving for at least 15 minutes. Some lived as long as 30 minutes in 10 per cent formaldehyde.

Monkeys and infected as well as normal guinea pigs, when not allowed to defend themselves, are eagerly bitten by the stegomyias. Rats, however, are seldom bitten by these mosquitoes, and bats have never been seen to be attacked when placed in a cage with hungry stegomyia females.

Transmission Experiments with Stegomyia calopus from Man to Animals.

Attempts were made to reproduce the appearances of yellow fever in the guinea pig by permitting the stegomyias to bite the animals after having fed upon the blood of yellow fever patients during the early stage of the disease. The procedure consisted in putting one arm of the patient into a cage containing 100 to 300 mosquitoes which had been hatched from the larvæ in captivity, and allowing the mosquitoes to feed until most of the females were engorged with blood. The cage containing the insects was then carefully kept in the shadow; sometimes the females were transferred to a separate cage. A glass of water containing a pea and a green twig was put into the cage, and each morning a slice of banana or papaya was placed upon the wire wall.

Since an enormous amount of labor would be required to test the infectivity of each female mosquito, summary experiments were made; that is, a dozen or more mosquitoes were allowed to bite one and sometimes two animals at the same time. The animals were then placed in separate cages and kept under daily observation for a period of at least 1 month, in case no infection took place within a shorter time. The tests for infectivity were usually made 2 weeks after the feeding on the patients, but sometimes earlier.

As the protocols show, positive leptospiral transmission was obtained in one of the following experiments.

Experiment 1 (Negative).—Aug. 5, 1918. 40 recently hatched females of *Stegomyia calopus* were allowed to become engorged by feeding on the right arm of Case 9 (G. C.), a severe case of yellow fever, admitted on the 3rd day of disease to the hospital. The patient recovered. Aug. 10. Sixteen females surviving. Eggs were found on the wet leaves in the glass. The sixteen mosquitoes surviving were allowed to feed on a very young normal guinea pig on that day; that is, 5 days after feeding on the patient's blood. All became engorged within the period from 12 m. to 2 p.m. The guinea pig was removed for observation into another cage. It remained well with normal temperature (38–39.5°C.) for 11 days, when it died of an intercurrent disease. There was no jaundice or change in the liver or kidney.

Experiment 2 (Positive Transmission).—Aug. 15. Twelve of the sixteen mosquitoes used in the above experiments still surviving. Larvæ in the glass. Aug. 28. Eight still surviving (23 days after feeding on the patient). A normal guinea pig was placed in the cage with these eight mosquitoes, which immediately attacked the animal, all becoming engorged. The guinea pig was removed to another cage and kept under observation. Temperature 38°C. Aug. 29, temperature 38°C.; Aug. 30, 40°; Aug. 31, 38.6°; Sept. 1, 39.3°; Sept. 2, 38°; Sept. 3, 37.5°; Sept. 4, 36°. Death occurred in 6 days.

Autopsy.—Moderately marked general jaundice. The lungs showed several small ecchymoses; the liver was yellowish brown and mottled; the kidneys were congested and showed ecchymoses; the stomach contained blood-stained food; the intestines showed numerous serous and mucous ecchymoses, and the stool was mixed with blood; the bladder was half full of yellowish brown urine which contained an abundance of albumin and casts; the adrenals were congested; the spleen, pancreas, and testes were apparently unchanged; the heart showed a few minute ecchymoses on the surface.

Microscopic Examination.—Examinations of emulsions of the liver, kidney, and blood for the leptospira were negative. The infectivity of the liver and kidney was tested on another guinea pig by injecting organ emulsions into the subcutaneous tissue. This animal died under the same symptoms in 7 days.

The leptospira was demonstrated in the kidney in small numbers, but not in the liver or blood.

Experiment 2 shows that the mosquitoes which sucked the blood of a yellow fever patient on the 3rd day of the disease were infective to the guinea pig after a period of 23 days. That they were unable to produce a typical infection when tested 5 days after the feeding is shown in Experiment 1.

Experiment 3 (Negative).—Aug. 5, 1918. Twenty-eight females were allowed to become engorged on the right arm of Case 22 (A. M.), a fatal yellow fever case, admitted to the hospital on the 4th or 5th day of the disease. The patient died on the 10th day with typical symptoms. Aug. 10. Twelve surviving. Aug. 12 (7 days after feeding). The mosquitoes were placed on a normal guinea pig. Several became engorged, and several were killed by the animal. This guinea pig showed no symptoms within the period of observation and was discarded.

Aug. 28. The surviving females were allowed to bite a normal guinea pig. The temperature rose to 39.9°C. on the 5th day but soon returned to normal and the animal showed no symptoms afterwards.

Experiment 4 (Doubtful).—Aug. 14, 1918, 4.15 p.m. A large number of stegomyias were placed on the left arm of Case 10 (M. N.), admitted on the 2nd day of the disease. 60 females which had become fully engorged were put into another cage. The patient died of typical yellow fever on the 7th day. Aug. 15, 9.30 a.m. Another feeding by the same mosquitoes on the same patient. Aug. 20. 34 surviving. Aug. 27 (13 days after feeding). 29 surviving. These were allowed to bite a normal guinea pig, and all became engorged. The guinea pig showed the following symptoms: Aug. 28, temperature 37.6°C.; Aug. 29, 38°; Aug. 30, 38.2°; Aug. 31, 39.5°; Sept. 1, 39.8°; Sept. 2, 40°; Sept. 3, 38.4°; Sept. 4, 38.1°; Sept. 5, 37.8°; Sept. 6, 38°; Sept. 7, 38.2°; Sept. 8, 38.1°. There was a trace of icterus in the scleras on the 8th and 9th days, which faded during the following day.

As the animal returned quickly to normal without further symptoms, a positive diagnosis was not possible, but it is probable that there was a mild infection.

Experiment 5 (Negative).—Aug. 14, 1918, 5 p.m. The right arm of Case 8 (R. V.), admitted on the 2nd day of disease, was put into a cage containing about 50 mosquitoes. Only five females sucked the blood within about 30 minutes. The case was severe but recovered. Aug. 15, 12 m. The feeding was repeated, 32 females becoming engorged immediately. Aug. 25. Many minute larvæ were found in the vessel containing water. Aug. 28, 10 a.m. The surviving females, thirteen in number, were allowed to feed on a normal guinea pig. All eagerly

engorged. The guinea pig was placed in a separate cage for observation. As no symptoms developed within a month it was discarded.

Experiment 6 (Negative).—Aug. 28, 1918, 12 m. 80 female stegomyias were allowed to engorge on Case 52, a girl of 10 years, whose brother had died in the hospital 4 days previously with yellow fever, but who was showing only a suspicion of icterus in the scleras which disappeared on the following day. The case was so mild that the patient never was confined to bed and left the hospital in 7 days. The temperature did not exceed 39°C. and remained at that point for the 1st day only. If the case was one of yellow fever it was extremely mild. Sept. 11. 23 females surviving. These were allowed to feed on a normal guinea pig; all became fully engorged immediately. The guinea pig was put into a separate cage for observation for 10 days. No symptoms developed and the animal was discarded after 3 weeks.

Transmission Experiments with Stegomyia calopus from Animal to Animal.

In earlier experiments⁸ on *Leptospira icterohæmorrhagiæ* transmission of the disease from one guinea pig to another by means of *Culex pipiens* was unsuccessful. The present study with *Leptospira icteroides*, however, brought out the fact that *Stegomyia calopus* is capable of transmitting the experimental disease resembling yellow fever from an infected to a normal guinea pig. The positive results are few, notwithstanding the numerous attempts made, but are sufficiently conclusive to establish the main point, that this mosquito may serve as an intermediary host of *Leptospira icteroides*. The term intermediary host, however, is not used here in the sense understood in the case of certain protozoan organisms, which require an extrinsic host in which to pass their life cycle, but denotes that a certain length of time is necessary for multiplication of the organisms to such numbers that the mosquito may transmit enough to produce infection. From the biological and cultural properties of the organism this hypothesis seems reasonable, though the possibility of a stage of development in the mosquito has not been excluded. The following experiments indicate the positive transmission of *Leptospira icteroides* from animal to animal by the bite of the stegomyia mosquito.

⁸ Noguchi, H., The survival of *Leptospira (Spirochæta) icterohæmorrhagiæ* in nature; observations concerning microchemical reactions and intermediary hosts, *J. Exp. Med.*, 1918, xxvii, 609.

Experiment 7 (Negative).—Aug. 4, 1918. 50 female stegomyias were engorged with the blood of a guinea pig which had been infected with the Case 1 strain of *Leptospira icteroides* 6 days previously. The animal was showing the organisms in the blood and had all the characteristic symptoms. Aug. 8. The mosquitoes fed on another guinea pig with beginning fever and albuminuria. The blood contained occasional leptospiras. Aug. 10. The mosquitoes which were used later in the experiments recorded below were allowed to bite a normal guinea pig on the 6th day after the first feeding. Only five mosquitoes attacked the animal. No symptoms followed the biting, and the guinea pig was discarded as negative on Sept. 13.

Experiment 8 (Positive).—Aug. 16 (12 days after the first and 8 days after the second feeding). Two normal guinea pigs were placed in the cage with the mosquitoes. Guinea Pig 544 was bitten by nineteen mosquitoes. The highest temperature was 39.6°C. on the 5th day, and the animal became mildly icteric on the 8th day. On the 12th day the temperature dropped to 37° and death ensued.

Autopsy.—Fatty degeneration of the liver, recent hemorrhagic spots in the lungs, semidigested bloody contents in the stomach and intestines, acute parenchymatous nephritis, and some ecchymoses in the lymph glands. The icterus was very slight. The leptospira was demonstrated under the dark-field microscope.

Guinea Pig 545 was bitten by nine other mosquitoes on the same day. The highest temperature was reached on the 6th day, but the animal gradually returned to normal without any icterus. It remained well for 24 days and was then subjected to a second infection with the same strain of the organism on Sept. 7. It proved refractory to the infection.

Experiment 9 (Negative).—The mosquitoes used in Experiment 8 were kept for another experiment, but within 5 days only eight of the twenty-eight still survived. These were allowed to feed on another normal guinea pig (No. 571) on Aug. 21. The animal showed no symptoms after the biting and was discarded as negative on Sept. 7.

Experiment 10 (Positive).—Aug. 13, 1918. In this series four guinea pigs (Case 1 strain) infected at different stages of the disease, 6th, 7th, 8th, and 9th days after the inoculation, all showing the symptoms, some intensely icteric, and some showing the leptospiras in the blood, were used to infect the mosquitoes in four separate cages. Three of the guinea pigs died subsequently, and one recovered. 142 engorged females in all were collected and put together in one cage for further experiments.

Aug. 21 (8 days after feeding on the infected guinea pigs). There were 83 mosquitoes surviving, and these were allowed to bite a normal guinea pig. All the mosquitoes sucked the blood eagerly. The protocol of this animal follows.

Guinea Pig 570.—Aug. 21. Bitten by 83 stegomyias. Temperature Aug. 22, 38.2°C.; Aug. 23, 38°; Aug. 24, 38.1°; Aug. 25, 39.8°; Aug. 26, 38°; Aug. 27, 37.2°; Aug. 28, 36.8°; Aug. 29, 37.6°; Aug. 30, 37°; Aug. 31, 36.2°; Sept. 2, 37.2°; Sept 3, 36.5°. Death occurred during the night 13 days after the animal had been bitten.

Autopsy.—Jaundice general but mild; hemorrhages in the lungs, stomach, and intestines; liver yellowish brown; kidney intensely congested, containing some minute ecchymoses, yellowish; bladder filled with yellowish brown urine with albumin and casts. Ecchymosis in the serosa and mucous membranes in general. Very few of the leptospiras in the kidney, none in the liver or blood.

Experiment 11.—To supplement the foregoing experiments, on Aug. 23, 1918, that is 2 days later, the same mosquitoes, twenty-five in all, were crushed in a mortar and emulsified in Ringer's solution. The emulsion was examined for the leptospira under the dark-field microscope and also smeared over the scarified surface of the skin of a normal guinea pig. Occasional specimens of leptospira were found in the emulsion after long search.

The guinea pig, No. 544 A, came down with the typical symptoms on Aug. 31; that is, 8 days after the inoculation. In the liver and kidney the leptospira was demonstrated in small numbers under the dark-field microscope.

This experiment conclusively proves that the leptospira was present in the body of the stegomyia mosquitoes which had been fed on the infected guinea pig and were capable of transmitting infection by their bite to another animal. The course of the infection produced by smearing the mosquito emulsion over the skin was more rapid than that caused by the bite.

Experiment 12 (Negative).—Some of the mosquitoes used in Experiment 10 which had caused a positive transmission of *Leptospira icteroides* after 8 days from the time of feeding on infected guinea pigs were kept another week. As already stated, twenty-five of these 83 mosquitoes were crushed on Aug. 23 for a supplementary confirmation (Experiment 11) of Experiment 10. Subsequently most of the remaining females laid eggs, and some perished. On Aug. 28, 7 days after Experiment 10, only thirteen were left. On that date a normal guinea pig (No. 633) was placed in the cage, and all the mosquitoes became engorged. The guinea pig was kept under daily observation for 13 days, but there was no suspicion of infection, and it was discarded on Sept. 10.

Experiment 13.—Aug. 11, 1918, 11 a.m. An infected guinea pig (Case 1 strain) showing the typical symptoms was placed in a mosquito cage. Twenty-four engorged females were collected and put into another cage. Aug. 14. Sixteen additional engorged females were also put into the cage, making the total 40. Aug. 21 (10 days later). A normal guinea pig was placed in the mosquito cage. The mosquitoes fed eagerly, all becoming engorged within 10 minutes. This animal (Guinea Pig 572) remained apparently well for 11 days. It was found dead on Sept. 2. At no time did the morning temperature exceed 38.4°C., which was about normal for this animal.

Autopsy.—A trace of jaundice throughout the body; liver highly degenerated and pale yellow; a few ecchymoses in the lungs; kidneys much congested. There was too scanty a quantity of urine in the bladder to examine for albumin. Diagnosis: probably a mild infection with *Leptospira icteroides*.

SUMMARY.

The foregoing experiments show that symptoms and lesions closely resembling those of yellow fever in man may be induced in guinea pigs by the bite of female stegomyias that have previously sucked the blood of a yellow fever patient or of an animal experimentally infected with *Leptospira icteroides*. With mosquitoes infected directly from a yellow fever patient the infectivity seems to become manifest after a longer period of incubation than with those infected with the animal blood. In the former, at least 12 days are said to be necessary before they become infectious, and this hypothesis seems to be borne out by the present experiment. On the other hand, the mosquitoes which were engorged with the infected blood of the guinea pig were found to be capable of transmitting the disease within 8 days after the feeding. This discrepancy may be explained by the fact that the number of leptospira existing in experimentally infected guinea pigs is far greater than that in human blood.

The frequency with which positive transmission by the stegomyia was obtained in both instances was very small indeed, in view of the number of mosquitoes employed. It appears that even under natural circumstances the percentage of mosquitoes that eventually become infected with the yellow fever microbe by sucking the blood may be very small. It has already been shown by previous investigators^{2,5} that to transmit yellow fever from a patient to a non-immune person requires from 0.1 to 2 cc. of blood at the height of disease. According to my estimate a female stegomyia may take up 0.01 cc. or even less. Apparently a mosquito occasionally becomes infectious by taking up the one or two organisms which happen to be circulating in the peripheral blood of man, and it is these occasionally infected few which carry the disease. It is not difficult to realize the extent of ever increasing danger from a constant supply of the microbic virus which an endemic center or an epidemic of yellow fever can provide. One infected mosquito may mean many patients, and the life of such a mosquito is usually longer than that of the persons whom it fatally infects.

Finally, it is of interest to note that the development and maintenance of *Leptospira icteroides* are indispensably associated with the blood constituent, the serum, and this is amply supplied by the blood-

sucking insect. The organism is one of the most fragile of all the pathogenic parasites and cannot survive the concurrence of other less fastidious organisms such as bacteria. The comparatively aseptic body cavity of the stegomyia⁹ furnishes a secure shelter for the parasite, which undoubtedly penetrates the zone of safety as soon as it is taken into the stomach of the insect. Unlike many other parasites this organism is capable of penetrating the intact skin or a bacteria-proof filter, and hence it is probably an easy matter for it to pierce the tissue of the visceral organs of the mosquito. Whether or not *Leptospira icteroides* can survive and multiply only in the body of *Stegomyia calopus* and not in other varieties or genera is yet to be determined.

Another interesting fact with regard to the extrinsic life of this organism is that it can multiply steadily at a temperature from 18–37°C. The optimum temperature, at which it remains viable for many months, is 26°. The climate in most of the tropical countries offers optimum conditions both for *Leptospira icteroides* and for the mosquito which carries and nourishes it.

⁹ This refers to the presence of bacteria and not certain higher plant parasites (yeast, moulds, etc.) or protozoa which have been occasionally found in stegomyia mosquitoes. These non-bacterial organisms may exert no adverse influence upon *Leptospira icteroides*.

CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

CHEMICAL SERIES. I.

N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID.

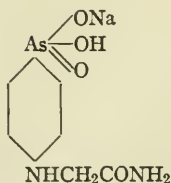
BY WALTER A. JACOBS, PH.D., AND MICHAEL HEIDELBERGER, PH.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 18, 1919.)

For a number of years the writers, in conjunction with Dr. Wade H. Brown and Dr. Louise Pearce, have been engaged in the synthesis of certain new types of organic arsenic compounds for the treatment of experimental trypanosome and spirochete infections. Although considerable of our effort has been given to the study of trivalent arsenicals our attention was at first confined to the development of compounds containing arsenic in the pentavalent form as presented by the arsonic acids. This group seemed best suited for the synthetic procedure which was employed and afforded the best opportunity for obtaining such information as to the relationship between chemical structure and biological action as would be useful for further work. Again it was felt that if a practicable and efficient drug could be found within this group it would go far toward eliminating certain of the well known practical disadvantages of the usual arseno compounds, for as an arsonic acid it should form soluble and stable salts and perhaps offer fewer of the preparative uncertainties than have been the experience with trivalent arsenicals.

In the course of these studies a number of substances have been prepared which have given interesting experimental results. However, those obtained with one in particular, the sodium salt of *N*-phenylglycineamide-*p*-arsonic acid



were such as to demand special attention. The simplicity of this compound, the ease of preparing it, its relatively inexpensive character, stability, and solubility, and its favorable biological behavior, warrant a publication of results. We wish therefore to present in conjunction with the following biological papers of Dr. Brown and Dr. Pearce, the facts as to the preparation and properties of the substance which will be appropriate in the present place. A somewhat fuller description of the substance and of related compounds has appeared elsewhere.¹

Phenylglycine-*p*-arsonic acid, and its homologue, *o*-methylphenylglycine-*p*-arsonic acid, have already been described in German Patent, No. 204,664. These substances, of interest solely as the source of the arsenophenylglycines which were obtained by their reduction, were readily prepared by the interaction of the sodium salts of the amino-arylarsonic acids and sodium chloroacetate. In our investigations we have found that a reaction of this type, with α -halogenacylamino compounds, XCH_2CONHR , instead of the α -halogen acids, XCH_2COOH , is capable of practically unlimited extension and has rendered possible the preparation of a new series of aromatic arsenic compounds. In these substances the free carboxyl group of the above glycines has been changed to the amide or substituted amide group.

N-Phenylglycineamide-*p*-arsonic acid,² prepared by the methods to be described in the experimental part, readily yields a colorless,

¹ Jacobs, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, 1919, xli, 1587.

² This substance and related compounds to be described elsewhere are covered by United States Patents, Nos. 1,280,119-1,280,127. Patents have also been applied for in foreign countries.

All discoveries made at The Rockefeller Institute for Medical Research are made freely available to the public, in accordance with the philanthropic purposes of the institution. In order to insure purity of product and protection against exploitation, it has been deemed necessary in certain instances to protect the

crystalline sodium salt which is extremely easily soluble in water, forming neutral solutions, which are perfectly stable. In fact a 10 per cent solution may be boiled a reasonable length of time without appreciable cleavage of ammonia or arsenic.

The materials required and the method of preparation are such that the large scale production of the substance should offer little difficulty.

EXPERIMENTAL.

N-(*Phenyl-4-Arsonic Acid*)-*Glycineamide* (*N*-*Phenylglycineamide-p-Arsonic Acid*).—Of the two methods used for the preparation of this substance that described first is more direct.

434 gm. of arsanilic acid were dissolved in 2 liters of normal sodium hydroxide solution. After the addition of 375 gm. of chloroacetamide the mixture was boiled under a reflux condenser for 45 minutes, the clear solution setting to a solid mass of the crude product on cooling. 75 cc. of concentrated hydrochloric acid were added to the cold mixture to hold any unchanged arsanilic acid in solution and the substance was then filtered off and carefully washed with cold water. For purification it was suspended in sufficient water to form a thin paste and carefully treated, with stirring, with 25 per cent sodium hydroxide solution until the acid was completely dissolved. The filtered solution was then treated with an excess of acetic acid whereupon the substance separated as minute, lustrous plates. After filtering, washing thoroughly, and drying, the yield was 300 gm.

The acid is very sparingly soluble in cold water but dissolves readily on heating. It separates from the hot aqueous solution in aggregates of long, thin plates. It is insoluble in methyl alcohol, acetone, or chloroform and sparingly in hot methyl or ethyl alcohol, but dissolves in boiling acetic acid. It is sparingly soluble in dilute

discoveries by patents. It is the purpose of the Institute to permit any drugs which may prove of practical therapeutic value to be manufactured under license by suitable chemical firms and under conditions of production which will insure the biological qualities of the drugs and their marketing at reasonable prices. Other than through the issuance of license, The Rockefeller Institute does not participate in any way in the commercial preparation or sale of the manufactured chemicals; and it receives no royalties or other pecuniary benefits from the licenses it issues.

hydrochloric acid but dissolves readily in the strong acid, its behavior showing it to be a weaker base than arsanilic acid. On boiling its solution in sodium hydroxide ammonia is evolved. When rapidly heated in an open capillary tube it darkens and softens at 280°C., but does not melt.

0.1405 gm. of substance; 12.35 cc. of N (22.0°C., 761 mm.).

0.3205 gm. of substance; 0.1832 gm. of $\text{Mg}_2\text{As}_2\text{O}_7$.

Calculated for $\text{C}_8\text{H}_{11}\text{O}_4\text{N}_2\text{As}$: N, 10.22 per cent; As, 27.33 per cent.

Found: N, 10.18 per cent; As, 27.59 per cent.

Sodium Salt.—The pure acid is suspended in enough water to form a thick paste and carefully treated with 25 per cent sodium hydroxide solution until completely dissolved and the solution reacts neutral to litmus. Two volumes of alcohol are then added, the pure sodium salt quickly separating as thin, nacreous plates. After filtering and washing with 85 per cent alcohol it is air-dried and then contains one-half molecule of water of crystallization. The sodium salt is extremely soluble in cold water, the solution reacting neutral to litmus. The apparatus, filter paper, water, etc., used in the preparation of this salt must be free from calcium; otherwise a precipitate of the insoluble calcium salt will contaminate solutions of the sodium salt.

0.3921 gm. of air-dry substance, at 100°C. *in vacuo* over H_2SO_4 ; 0.0117 gm. loss.

Calculated for $\text{C}_8\text{H}_{10}\text{O}_4\text{N}_2\text{AsNa} \cdot \frac{1}{2} \text{H}_2\text{O}$: H_2O , 2.95 per cent.

Found: H_2O , 2.98 per cent.

Anhydrous: 0.1503 gm. of substance; 12.45 cc. of N (25.0°C., 762 mm.).

0.2300 gm. of substance; 0.1195 gm. of $\text{Mg}_2\text{As}_2\text{O}_7$.

Calculated for $\text{C}_8\text{H}_{10}\text{O}_4\text{N}_2\text{AsNa}$: N, 9.46 per cent; As, 25.32 per cent.

Found: N, 9.52 per cent; As, 25.08 per cent.

The potassium and ammonium salts were prepared in the same way as the sodium salt and form thin, glistening, hexagonal, microscopic platelets. On adding a calcium chloride solution to a solution of the sodium salt the calcium salt gradually separates as microscopic wedge-shaped prisms, containing no water of crystallization. Magnesia mixture causes no precipitate in the cold, but on warming the magnesium salt separates as a microcrystalline powder. Heavy

metal salts give immediate precipitates, the silver salt forming aggregates of thin, microscopic needles.

N-Phenylglycineamide-*p*-arsonic acid was also prepared as follows from *N*-phenylglycine methyl ester-*p*-arsonic acid by the action of ammonia.

N-(*Phenyl-4-Arsonic Acid*)-*Glycine Methyl Ester*.—40 gm. of *N*-phenylglycine-*p*-arsonic acid³ were treated with 120 gm. of dry methyl alcohol and 4 gm. of concentrated sulfuric acid. The mixture was boiled under a reflux condenser for 2 hours. The ester separated on cooling and scratching, the precipitation being completed by the addition of water. The filtered, washed, and dried product weighed 38 gm. It can be recrystallized from hot water or hot 95 per cent alcohol, separating from the former as microscopic needles and thin plates. It is very sparingly soluble in cold water, cold alcohol, or boiling acetone, and is fairly easily soluble in methyl alcohol, especially on warming. When rapidly heated it softens and darkens above 200°C. and decomposes at about 285°.

0.1560 gm. of substance; 7.0 cc. of N (21.0°C., 747 mm.).

0.3135 gm. of substance; 0.1665 gm. of $Mg_2As_2O_7$.

Calculated for $C_9H_{12}O_5NAs$: N, 4.84 per cent; As, 25.94 per cent.

Found: N, 5.12 per cent; As, 25.63 per cent.

The ester was converted into the amide as follows: 10 gm. of the ester were slowly added, with stirring, to 30 cc. of well chilled, concentrated ammonia. At first a thick paste of the ammonium salt of the ester was formed, but on allowing the mixture to rise to room temperature the reaction proceeded with formation of a clear solution. After 24 hours the excess of ammonia was removed, preferably *in vacuo*. On diluting with water, filtering, and acidifying with acetic acid, *N*-phenylglycineamide-*p*-arsonic acid separated in characteristic form. This was purified as described above and was identical in every way with the product obtained by the direct method. The yield was 80 per cent of the theory.

0.1297 gm. of substance; 11.5 cc. of N (19.5°C., 742 mm.).

Calculated for $C_8H_{11}O_4N_2As$: N, 10.22 per cent.

Found: N, 10.11 per cent.

³ German Patent, No. 204,664.

CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

BIOLOGICAL SERIES. I.

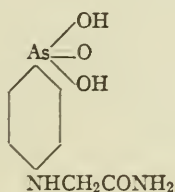
THE TOXIC ACTION OF N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID.

BY WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 18, 1919.)

The chemotherapeutic investigations which have been in progress in these laboratories for several years have yielded a number of substances of striking activity in the treatment of experimental infections such as those produced in laboratory animals by various species of trypanosomes, the spirochetes of relapsing fever, and *Treponema pallidum*. Among the first of these substances was the amide of *N*-phenylglycine-*p*-arsonic acid, a description of which is given by Dr. Jacobs and Dr. Heidelberger, in the chemical series of these papers.¹ It may be said that while *N*-phenylglycine-*p*-arsonic acid, described in German Patent, No. 204,664, is a substance of practically no importance in the treatment of these infections, arsenophenylglycine, produced from it by reduction, was among the earliest of the highly active trypanocidal agents. The amide of this acid which has the structural formula



was first made and studied in the fall of 1915 with results which at once opened the way to the development of a number of important substances which will be dealt with in subsequent papers.

¹ Jacobs, W. A., and Heidelberger, M., *J. Exp. Med.*, 1919, xxx, 411.

Methods of Employing N-Phenylglycineamide-p-Arsonic Acid.

The amide of *N*-phenylglycine-*p*-arsonic acid, or A 63 as it was designated on our lists, presents no difficulty in the way of its use for purposes of animal experimentation. In the form of the monosodium salt, the drug is readily soluble in water in concentrations as high as 50 per cent and when injected directly into the tissues or into the body cavities of animals, it is readily absorbed and produces but slight irritation or local injury.

Preparation of Solutions.—Solutions of A 63 may be prepared by dissolving the monosodium salt in sterile distilled water, or in case the acid is used, the requisite amount of sodium hydroxide (0.37 cc. of *N* sodium hydroxide per 0.100 gm. of drug) to form the monosodium salt should be added slowly with stirring. The product then dissolves without difficulty.

Administration.—This drug may be administered to animals by almost any route which is convenient and has been used subcutaneously, intramuscularly, intraperitoneally, intravenously, and *per os* with no especial disadvantage following its use by any except the last named route.

Measurement of Doses.—The measurement of doses was accomplished in one of three ways. With the smaller animals, a stock solution of the drug was prepared from which individual doses for mice were measured by the use of a Fournier tuberculin syringe graduated in hundredths of a cubic centimeter; in the case of rats and guinea pigs, doses were measured with standardized pipettes. Finally, doses for larger animals such as rabbits and monkeys were always weighed and prepared separately. The values given in all cases refer to amounts of the monosodium salt which was used almost exclusively. These figures may be transposed into equivalents of the acid by the use of the factor 0.9 if desired.

Minimum Lethal Dose.

Although A 63 contains 24.57 per cent of arsenic, its toxicity for laboratory animals is comparatively low. The lethal dose of the drug varies between the extremes of 0.75 and 2.75 gm. per kilo of body weight for the different animal species in which it has been tested and for different routes of administration.

Mice.—The tolerance of mice is particularly good, as is indicated by the figures given in Table I which represents the combined results of several experiments carried out with mice. From the data at our disposal, we would place the minimum lethal dose for mice at 2.5 to 2.75 gm. per kilo of mouse when given subcutaneously, 2 to 2.25 gm. given intraperitoneally, and 2 gm. when given intravenously. These figures show a reasonably close agreement in the toxicity of the drug when administered by these routes, and the results obtained from successive experiments were comparatively uniform. Some mice survive even higher doses than those shown in the table, and conversely an occasional mouse may succumb to lower doses than those given as the lethal or minimum lethal dose; but we have had no

TABLE I.

Lethal Effects Obtained from the Administration of a 5 Per Cent Solution of N-Phenylglycineamide-p-Arsonic Acid to Mice in Doses Equivalent to 1.75 to 2.5 Gm. per Kilo of Mouse.

Dose per kilo.	Subcutaneous injection.		Intraperitoneal injection.		Intravenous injection.	
	No. of mice used.	No. died.	No. of mice used.	No. died.	No. of mice used.	No. died.
<i>gm.</i>						
2.5	2	0	5	1	2	2
2.25	4	0	21	5	4	1
2.0	4	0	25	2	4	1
1.75	4	0	8	0	4	0

deaths resulting from doses of the drug below 2 gm. per kilo of mouse.

Rats.—The resistance of white rats to the toxic action is lower and more irregular than that of any other animal with which we have worked. When the drug is administered intraperitoneally, doses as small as 0.75 gm. per kilo are sufficient to cause death in a fair percentage of animals and yet some rats will survive as much as 1.75 gm. per kilo. The results obtained from subcutaneous administration are distinctly better; the minimum lethal dose rises to 1 gm. per kilo and the action of the drug is definitely more constant, as may be seen by an examination of the results from the two experiments incorporated in Table II.

TABLE II.

Lethal Effects Obtained from the Subcutaneous Administration of a 10 Per Cent Solution of N-Phenylglycineamide-p-Arsonic Acid to Rats and from the Intraperitoneal Administration of a 3 Per Cent Solution.

Dose per kilo.	Subcutaneous injection.		Intraperitoneal injection.	
	No. of rats used.	No. died.	No. of rats used.	No. died.
<i>gm.</i>				
1.50	4	4	5	4
1.25	4	1	5	3
1.00	9	6	9	7
0.90	5	0	5	2
0.75	5	0	5	3
0.60			5	0
0.50			5	0

Guinea Pigs.—Contrary to the opinion which is generally held as to the tolerance of guinea pigs for arsenicals, these animals withstand relatively large doses of A 63 and the resistance of individual animals appears to be fairly uniform. The lethal dose found for the drug was 1.5 gm. per kilo of body weight whether given subcutaneously or intraperitoneally (Table III).

TABLE III.

Lethal Effects Obtained from the Administration of a 20 Per Cent Solution of N-Phenylglycineamide-p-Arsonic Acid to Guinea Pigs.

Dose per kilo.	Subcutaneous injection.		Intraperitoneal injection.	
	No. of guinea pigs used.	No. died.	No. of guinea pigs used.	No. died.
<i>gm.</i>				
1.75			4	1
1.50	4	3	9	3
1.40	4	0	4	0
1.25	4	0	4	0

Rabbits.—A great deal of time has been devoted to the study of the toxic action of A 63 in rabbits. The major part of this work was carried out by intravenous administration of the drug but the effects

of subcutaneous, intramuscular, and *per os* administrations have all been investigated to some extent. The solutions used in the experiments varied between concentrations of 5 and 50 per cent, partly for the purpose of studying the effects of the use of different volume doses and partly for the purpose of determining the influence of such factors as rate of administration and concentration of solutions upon the action of the drug.

It was found that as much as 20 cc. per kilo of a 5 to 10 per cent solution could be injected intravenously into rabbits about as rapidly as one wished, and that in general, solutions of low concentration (5 to 10 per cent) were better borne than those of a higher concentration. The effects of concentration were less evident, however, when the drug was administered by other routes, and more concentrated solutions were employed as a means of reducing the volume of fluid which had to be used.

Under the conditions described, the minimum lethal dose for rabbits was found to be 0.75 to 0.9 gm. per kilo of body weight when given intravenously and 1.1 gm. given either subcutaneously or intramuscularly, with lethal effects as indicated by the experiments recorded in Table IV.

TABLE IV.

Lethal Effects Obtained from the Intravenous Administration of a 5 to 10 Per Cent Solution of N-Phenylglycineamide-p-Arsonic Acid to Rabbits and from the Subcutaneous or Intramuscular Administration of 50 Per Cent Solutions.

Dose per kilo.	Subcutaneous injection.		Intramuscular injection.		Intravenous injection.	
	No. of rabbits used.	No. died.	No. of rabbits used.	No. died.	No. of rabbits used.	No. died.
gm.						
1.25	8	6	4	3		
1.1	4	4	4	2		
1.0	3	0	3	0	6	3
0.9					12	4
0.75					10	1

Our experience in giving this drug to rabbits by mouth may be recited very briefly. The drug was given in solution by means of

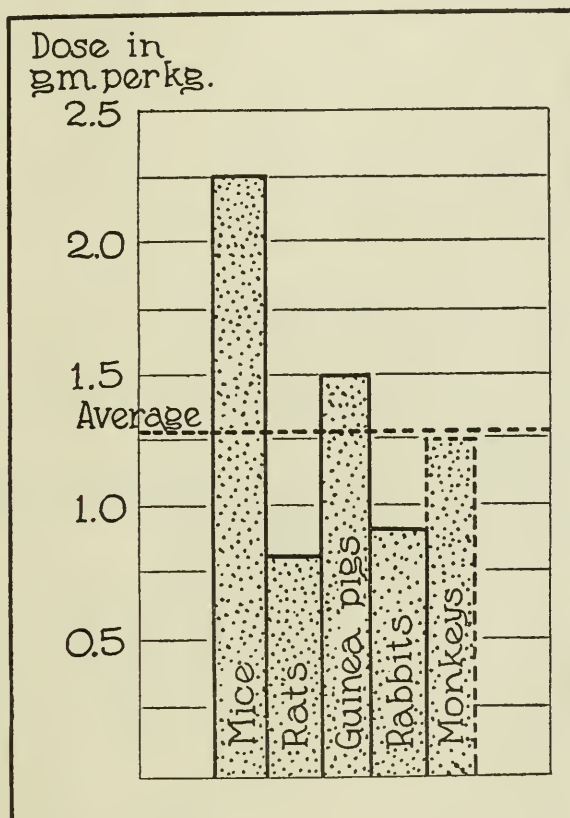
a stomach tube following the administration of a small dose of bicarbonate of soda. In all, there were only seven rabbits which received the drug in this way. Two of them were given doses of 1.25 gm. per kilo of body weight, four were given 1 gm., and one was given 0.75 gm. The last of these animals showed no ill effects from the drug and only two of the others actually succumbed to its action, both of which received a dose of 1 gm. per kilo. One of these rabbits lived 4 days and the other 24 days after the administration of the drug. It appeared certain, however, that none of the other animals would recover completely and they were killed at different times for purposes of pathological examination. As the matter stands, therefore, we are hardly justified in attempting to fix the toxic limits of A 63 when given *per os*.

Monkeys.—Lastly, we have used two monkeys (*Macacus rhesus*) in studying the toxic action of A 63. The first monkey, a female weighing 2,325 gm., was given an initial dose of 0.75 gm. per kilo of body weight. The animal was observed for 5 days during which time no ill effect from the drug could be detected, and a second dose of 1 gm. per kilo was given with a like result. 4 days later, the animal received its third dose which was 1.25 gm. per kilo. Following this dose, the only evidence of intoxication noted was possibly a slight loss of weight (75 gm.). At the end of 3 weeks, the weight of the animal was 2,450 gm., and a fourth dose of the drug was given—this time 1.5 gm. per kilo, or double the initial dose. The monkey showed slight signs of intoxication lasting for a few days, followed by rapid recovery. The animal was kept under observation for 3 months and was then killed for pathological purposes.

The second monkey used was likewise a female *rhesus* weighing 2,650 gm. This monkey was given an initial dose of 1.25 gm. per kilo intravenously. There was a loss of weight in this animal amounting to 175 gm., but no other evidence of intoxication developed, and after waiting 1 week, a second dose of 1.5 gm. per kilo was given; death followed within 24 hours.

These two experiments are cited merely to show that monkeys possess a degree of tolerance for A 63 comparable with that of other animals and that they react to the drug in much the same way.

Represented graphically, the values obtained for the minimum lethal dose of this drug in the five species of animals studied would form a curve such as that given in Text-fig. 1. While these values are not strictly comparable on account of differences in the mode of



TEXT-FIG. 1. Comparative magnitudes of the minimum lethal dose of *N*-phenyl-glycineamide-*p*-arsonic acid for different animal species. The dose given for monkeys is an approximate estimate.

administration used, they will serve to indicate the relative magnitude of the toxic dose for different animal species. The mean toxic dose for this group of animals is found to be approximately 1.28 gm. per kilo; the maximum variation from this mean is as 1:1.75 and the extremes compare as 1:2.8.

Symptoms and Course of the Intoxication.

The symptom-complex of the intoxication produced by *N*-phenylglycineamide-*p*-arsonic acid in laboratory animals is characterized by two groups of phenomena, one nervous and the other nutritional in character. The most prominent of these symptoms appear at an early period of the intoxication, as pronounced tremors with incoordination of movements, or in extreme cases, as clonic spasm, usually associated with some weakness or complete prostration. With remission of these early symptoms in mice, a tic develops which is characterized by peculiar jerky movements of the head and occasionally by the continuous circling movements of dancing mice. Nutritional disturbances are indicated chiefly by a loss of appetite, more or less weakness, loss of weight, and occasionally by diarrhea. Altogether the picture is one previously recognized and described as characteristic of the toxic action of a number of pentavalent arsenicals, particularly of arsacetin, dichlorophenylarsonic acid, and aminohydroxyphenylarsonic acid.

These symptoms are by no means constant either as to the frequency or the intensity of their occurrence in different animal species or in individual animals of the same species.

Mice.—In mice symptoms of intoxication occur only following relatively large doses of the drug (1.75 gm. per kilo and above) and even then are not of constant occurrence. In the most marked cases, mice show violent muscular tremors on being disturbed and marked locomotor incoordination. After 2 or 3 days, these symptoms disappear entirely or gradually give place to the tic described, which in turn rarely persists for more than a week. Disturbances of this general character appear to be more pronounced following subcutaneous and intravenous administrations of the drug than after intraperitoneal administration.

Mice also show some nutritional disturbances for 2 or 3 days following toxic doses of the drug but make a rapid recovery with an increase in weight above their normal level and remain subsequently in excellent condition.

Rats.—Rats are, on the whole, the most sensitive animals to this drug and individual idiosyncrasy is quite pronounced among them.

Nevertheless, toxic symptoms in these animals are fairly well confined to doses within what we regard as the lethal range. While some rats show no toxic symptoms at all, in others, tremors, incoordination, convulsions, weakness, and emaciation reach a most extreme grade and progress to a lethal termination, recovery under such conditions being very rare.

Guinea Pigs.—Guinea pigs on the other hand are quite resistant to A 63. They show very slight evidence of intoxication either of a nervous or of a nutritional character from doses below the lethal level. The dose that is survived is usually well borne and even lethal doses produce comparatively slight tremors, unsteadiness, weakness, or loss of weight.

Rabbits.—The reaction of rabbits to A 63 is rather irregular. By whatever route the drug is administered, doses above 0.5 to 0.6 gm. per kilo of body weight cause some loss of appetite with an initial loss of weight which is in proportion to the size of the dose used. With doses of 0.75 to 0.9 gm. per kilo of body weight, the loss of weight in surviving animals may reach as much as 200 to 300 gm. and is associated with some degree of weakness. This loss of weight, however, is quickly regained, leaving no symptomatic evidence of intoxication. Nervous phenomena of the exact type described in mice and rats do not occur in rabbits. Instead, rabbits manifest some hypersensitiveness in the milder cases of intoxication, while in extreme cases incoordination develops with a tendency to clonic spasms, and there is eventual loss of muscular control or even paralysis. As in the case of the rat, pronounced nervous symptoms in rabbits nearly always portend a lethal outcome so that these phenomena cannot be said to occur as symptoms of a sublethal intoxication.

When toxic doses are given to rabbits *per os*, the drug produces symptoms which are not observed when it is given by other routes, the characteristic feature of which is a marked abdominal distention with flatulence as a result of atony of the colon. Associated with this condition, there is a loss of appetite, diarrhea, progressive loss of weight, and weakness. The condition is extremely persistent and recovery, if it occurs at all, is problematical.

Monkeys.—In our limited experience with monkeys, we have observed no symptoms of intoxication other than slight loss of weight and some weakness as previously recorded.

Having described the symptoms of intoxication produced by A 63, we shall now take up the question of the time element in these toxic reactions. In mice symptoms of intoxication are usually apparent within 24 hours after the administration of the drug but may be delayed until the 2nd day. Death also occurs relatively early in these animals and is rare after 48 to 72 hours. Symptomatic recovery is relatively prompt.

Rats, on the other hand, usually show no symptoms of intoxication until the 2nd or 3rd day except with very large doses of the drug. Out of twenty-eight rats that died as a result of intoxication with doses below 1.5 gm. per kilo of body weight, the earliest death occurred 3 days after the administration of the drug, the latest deaths 10, 11, and 14 days, while the average period was 6 days. Recovery in rats is also a slow and uncertain process.

Symptoms of intoxication in guinea pigs usually make their appearance on the 2nd day following the administration of the drug. Among the deaths recorded in our series from doses below 2 gm. per kilo, the earliest occurred 2 days and the latest 7 days after the administration of the drug with an average period of 4 days survival. Recovery in guinea pigs is usually prompt and complete.

Of the rabbits which died after intravenous injections of a single dose of A 63, one with the smallest dose (0.75 gm.) died in almost exactly 24 hours, while the longest survival was 6 days and the average 2.6 days. The minor symptoms of intoxication in rabbits are not apparent as a rule until the 2nd day, while the graver symptoms occur more promptly. As previously indicated, symptomatic recovery in the rabbit is usually prompt and complete.

Pathology of the Intoxication.

The pathological changes produced in the animal organism by toxic doses of A 63 constitute an important phase of its toxicologic action. Viewed from the standpoint of the bearing of these changes upon the possible usefulness of the drug as a therapeutic agent, this phase of the subject resolves itself largely into a consideration of organic injury and recovery therefrom.

Local Effects.

As we have already indicated, the injury produced by the drug at the site of injection is almost negligible. Full toxic doses may be injected into the veins of animals even in saturated solution, with practically no local reaction. When a 50 per cent solution of the drug is injected into the subcutaneous tissues of animals, a slight edema develops at the site of the injection but clears up almost immediately, leaving such slight evidences of tissue reaction as are barely recognized from external examination. If given intramuscularly in amounts small enough to obviate mechanical laceration of the tissues, the reaction which follows is likewise very mild and is of essentially the same character as that which follows subcutaneous administration.

Systemic Effects.

The effects produced by the drug upon the organism as a whole are of much more importance than those of a purely local character. These effects are divisible into major and minor phases of pathological action, the details of which differ somewhat in different animal species as well as with the amount of the drug used and the route of administration employed. In general, however, the changes seen during the early stages of the intoxication consist in moderate vascular dilatation and congestion with a few scattered petechial hemorrhages, occasional effusions into the serous cavities, and widespread cellular degenerations or even necrosis in some organs. The organs which share most prominently in these changes are the kidneys and adrenals, and the cardiovascular system, with an uncertain involvement of the central nervous system, the gastrointestinal tract, the blood, and blood-formative organs; the changes produced in other organs appear to be of minor importance. Animals which survive the intoxication rarely show lesions in organs other than the kidneys and myocardium.

Kidneys.—The central feature of the pathological action in all animals is the injury to the kidneys. In acute poisoning, the kidneys are somewhat enlarged and tend to be pale except for the presence of more or less congestion or even hemorrhage in the boundary zone

which may extend outward along the medullary rays to the capsular surface. The glomerular tufts are usually swollen, the capillaries congested, and the covering epithelium is degenerated. Degeneration or even necrosis of the tubular epithelium is rather widespread but occurs chiefly in the ascending loops of Henle and the convoluted tubules, particularly those located along the medullary rays and the outer portion of the cortex. The interstitial elements of the kidney are, as a rule, much less affected except when overwhelming doses of the drug are given.

As serious as these injuries appear to be in the acute stages, recovery is remarkably satisfactory in most animals. Reaction of the tissue elements sets in promptly and restoration of the injured parts is accomplished within a short period of time and with comparatively slight scarring or distortion of the architecture except where the initial destruction was unusually extensive. In these cases, the marks of injury are found in a growth of connective tissue and in round celled infiltrations extending along the medullary rays and through the outer portion of the cortex, while a few glomeruli show shrunken tufts and thickened capsules, all of which gives a granular surface to the kidneys.

Adrenals.—Evidences of injury to the adrenals consist in swelling, with some congestion of the cortical vessels and the occasional presence of focal hemorrhages in the cortex. In guinea pigs, the later changes are manifested by a decrease in the cortical pigmentation with well marked degenerative changes in the cortex and medulla or even necroses in the midcortical zone. Here again recovery appears to take place fairly promptly.

Cardiovascular System.—The most pronounced effects of A 63 upon the cardiovascular system appear during the early stages of the intoxication. There is a moderate vascular dilatation and congestion throughout the body and a few petechial hemorrhages may occur in any of the organs. The sites of chief importance are the kidneys, the adrenals, and heart in which occasional foci of hemorrhage are found beneath the epicardium and the endocardium as well as in the myocardium itself. In several instances, hemorrhages from the meningeal vessels have been observed, either in the region of the torcular or in the retroorbital tissues.

Animals which survive the acute poisoning show little evidence of either congestion or hemorrhage, but in their stead one finds an accumulation of more or less fluid in the serous cavities, perirenal edema, and in rare instances an increased tension of the cerebrospinal fluid. These conditions we regard as at least indicative of vascular injury. In addition to such changes as these, the myocardium shows a moderate fatty degeneration with occasional areas of fibrosis. On the whole, the pathological effects of A 63 upon the cardiovascular system are not pronounced and the conspicuous congestion, hemorrhage, and degeneration so often observed with arsenicals are largely absent from the changes which characterize the action of this drug.

Central Nervous System.—From the symptoms of the toxic action of A 63, one might expect the central nervous system to share the position of chief pathological importance with the kidneys and cardiovascular system. Such changes as occur, however, are rather obscure and difficult of determination in laboratory animals. We have already mentioned the occurrence of congestion of the meningeal vessels and occasional hemorrhages together with an increased tension of the cerebrospinal fluid. Histologically, the chief evidences of injury are associated with the choroid plexus and with the small and medium vessels of the meninges and brain. The plexus itself shows definite degeneration in some animals and the tissues immediately surrounding the vessels are somewhat edematous. Apart from these changes, however, we have been able to demonstrate only minor degrees of cellular degeneration of a rather indefinite character and of uncertain extent.

Blood and Blood-Formative Organs.—While the action of A 63 upon the blood and blood-formative organs forms an important feature of the effect of the drug, this action is perhaps more physiological than pathological and hence there is little that can be said in the present connection.

Poisoning with the drug produces an initial destruction of red blood cells and an accumulation of blood pigments in the spleen, liver, and bone marrow. The formative centers, especially those of the spleen, show degeneration or even necrosis after the administration of very large doses of the drug, but following these changes, there is an extremely active hyperplasia in which all elements participate.

This is most strikingly shown by a myeloid metaplasia in the spleen of rats and mice which is characterized by the presence of large numbers of megacaryocytes. These changes alone, however, might mean very little, since such reactions are easily excited in mice and rats.

The effect of A 63 upon other organs of the body is relatively inconsiderable. In the liver, parenchymatous and fatty degeneration are usually but not constantly present, and in exceptional instances isolated cells or groups of cells here and there show necrosis. As a rule, these changes clear up very quickly leaving nothing to indicate the existence of any previous injury.

Lesions of the gastrointestinal tract are likewise inconstant and of uncertain significance. During the early stages of the intoxication, a catarrhal condition of the entire tract is not infrequently observed but is rarely of serious degree. An exception to this rule is found in animals which have been given the drug by mouth. These animals always show a pronounced and persistent colitis with a lesser degree of involvement of the stomach and small intestine. This, however, is clearly referable to the mode of administration of the drug.

Apart from a knowledge of the character of the lesions which may be induced by this drug, the features of chief interest from the pathological point of view are the promptness with which repair is accomplished and the fact that, as the dose of the drug used falls below the level of the lethal dose, the extent of the resulting injury rapidly diminishes until, with doses only slightly below the level of the lethal dose, organic injury is barely demonstrable. These features of the pathological action of A 63 are both unusual and of considerable practical importance in their bearing upon the use of the drug for therapeutic purposes.

Tolerance of Repeated Doses.

The facts presented thus far have dealt entirely with effects produced by the administration of single doses of the drug, which brings us to a consideration of the reaction of the animal organism to the use of repeated large doses. This phase of drug action is bound up to a large extent with two conditions, first, the time during which the drug remains in the animal body in a form capable of exercising a

toxic effect, and second, the character and duration of the effect produced, whether functional or organic.

In attempting to arrive at some idea of the time during which A 63 remained biologically active after being administered to animals, we made use of two types of experiments—one based upon protection against infection and the other upon superposition of fractional parts of toxic doses. In general, it was found by experiments of the first class, that the protection afforded such animals as mice against infection with *Trypanosoma brucei* was practically nil 24 hours after the administration of as much as twice the dose of the drug which was capable of curing a 24 hour infection of the same organism (0.5 gm. per kilo). With rats and with rabbits on the other hand, some degree of protection still existed at the end of 48 hours. In the case of the rabbit, four animals which were inoculated with *Trypanosoma brucei*, 48 hours after having received an intravenous injection of 0.5 gm. of A 63 per kilo, gave the following results: One rabbit showed no protection, two were permanently protected, and the fourth showed an incubation period of 21 days as contrasted with an incubation period of 9 days in controls which received the same dose of organisms at the same time.

From these experiments it appeared that retention of the drug in a biologically active form was a variable condition both specifically and individually, but that in animals such as the rat and the rabbit, there was the possibility of the drug's remaining in the body for 48 hours or even longer in a condition in which it might still be capable of exerting toxic effects. To test this further, we carried out a few experiments on the effects of the administration of fractional parts of a toxic dose at various intervals of time. Without going into the details of these experiments, we may say that it was found that when the toxic dose of A 63 for rabbits (0.75 gm. per kilo) was divided into three equal parts administered at intervals of 24 hours, the toxic effect approached that produced by the administration of the entire amount of drug at one time. This, of course, might be interpreted either as evidence of an accumulation of drug or as evidence of superposition of effects notwithstanding the fact that no toxic effect could be recognized from the administration of a single such fractional part of the toxic dose.

Combining these facts with what we had already learned in regard to the general reaction of animals to single doses of A 63, we undertook some experiments intended to determine the tolerance of mice and of rabbits to prolonged repetition of large doses of the drug and something of the interval at which such repetitions might be carried out successfully. The results obtained with one series of mice are summarized in Table V.

These mice were given an initial dose of 2 gm. of A 63 per kilo of body weight injected into the peritoneal cavity. The injections were repeated at weekly intervals and the dose was progressively increased up to 3 gm. per kilo. The sixth and last dose of 2.5 gm. per kilo was

TABLE V.

Tolerance of Mice to Intraperitoneal Administrations of Increasing Doses of N-Phenylglycineamide-p-Arsonic Acid Given at Weekly Intervals.

No. of injection.	Dose per kilo.	No. of mice used.	No. intoxicated.	No. died.
	<i>gm.</i>			
1	2.00	15	5	2
2	2.25	13	0	0
3	2.50	13	2	2*
4	2.75	11	0	0
5	3.00	11	0	0
6†	2.50 (i.v.)	9	3	3

* These two mice were toxic after the first dose.

† The last injection was given intravenously.

then given intravenously. Five of the fifteen mice showed symptoms of intoxication following the administration of the first dose and two of these died. Two others died after receiving the third dose (2.5 gm.) but the remaining eleven mice survived the administration of 3 gm. per kilo with no evidence of intoxication, indicating a definite increase in their tolerance to intraperitoneal administrations of the drug. When the route of administration was changed, these animals still showed a resistance to the drug slightly greater than that of normal mice.

Experiments of a similar character were carried out with rabbits. In this case, three routes of administration were used—subcutaneous, intramuscular, and intravenous. The initial doses were placed at

what was regarded as comparable levels for the various routes of administration; *i.e.*, 0.75 gm. per kilo intravenously and 1 gm. per kilo given either subcutaneously or intramuscularly. Animals of the subcutaneous and intramuscular series received their last dose intravenously and those of the intravenous series were divided into two

TABLE VI.

Tolerance of Rabbits to Increasing Doses of N-Phenylglycineamide-p-Arsonic Acid Given at Weekly Intervals, (A) Subcutaneously, (B) Intramuscularly, and (C) Intravenously.

No. of injection.	Dose per kilo.	No. of rabbits used.	No. intoxicated.	No. died.
	<i>gm.</i>			
A 1	1.00	3	0	0
2	1.00	3	0	0
3	1.10	3	0	0
4	1.25	3	0	0
5	1.35	3	0	0
6*	1.10	3	0	0
B 1	1.00	3	0	0
2	1.00	3	0	0
3	1.10	3	0	0
4	1.25	3	0	0
5	1.35	3	0	0
6*	1.10	3	0	0
C 1	0.75	10	2	1
2	0.75	9	1	0
3	0.90	9	0	0
4	1.00	9	1	1
5	1.10	8	0	0
6*	1.35	8	1	1

* The last injections of Series A and B were given intravenously; in Series C, four rabbits were injected subcutaneously and four intramuscularly.

groups one of which was given the last dose subcutaneously and the other intramuscularly. The results of these experiments are given in Table VI.

Following the first dose, one rabbit of the intravenous series was extremely toxic and died within 24 hours. A second rabbit was slightly toxic but all the others remained in good condition. No

other toxic manifestations developed until the fourth dose of the series was given, when one rabbit of the nine which received the dose of 1 gm. per kilo intravenously became toxic and died after 17 days. The fourteen remaining rabbits were carried through to the conclusion of the experiment with no symptoms suggestive of a harmful effect other than slight fluctuations in weight.

The dose in the intravenous series was raised progressively from 0.75 gm. per kilo of body weight to 1.1 gm., which in our experience is almost uniformly fatal when given to normal rabbits, with the loss of only two out of the ten rabbits; the eight rabbits which received the dose of 1.1 gm. survived with no evidence whatsoever of intoxication. These eight rabbits were then given a dose of 1.35 gm. of A 63 per kilo of body weight either subcutaneously or intramuscularly with one death following an intramuscular injection. The other rabbits remained entirely normal and continued to gain weight. With the subcutaneous and intramuscular series, the dose was raised from 1 gm. per kilo of body weight to 1.35 gm. with no toxic developments, and all these animals were then given 1.1 gm. of the drug by the intravenous route. In this case, the weights declined slightly but there was no other sign of intoxication. It is to be noted that all three groups of rabbits were carried to a point where they not only survived doses which are ordinarily fatal but that when a normally lethal dose was given by a route other than that to which they had been accustomed, only one of the fourteen rabbits succumbed, while the others showed little or no ill effects.

At the conclusion of this experiment, the entire group of rabbits was killed for pathological examination. On opening these animals, there was a strong odor of garlic—a condition never noted in an animal which had died from a single dose of the drug. Pathologically, the only changes found were small subcutaneous abscesses or abscesses in the lumbar muscles of animals which had received repeated injections in the same regions, a slight fatty degeneration of the myocardium with a few patches of fibrosis, and chronic nephritis. The injuries to the heart muscle and to the kidneys were definitely more pronounced in the rabbits of the intravenous group than in the others. With reference to the kidneys of this group, we would classify them as two with slight lesions, two with moderate lesions, and four with pronounced lesions.

The total amount of drug received by each of these rabbits during a period of 36 days was 5.85 gm. per kilo in one group and 6.8 gm. per kilo in the other, or 1.437 to 1.67 gm. of arsenic administered within a period of 5 weeks. Finally, it should be recalled that the toxic dose of A 63 for rabbits is lower than that for any other animal with which we have worked except the rat, and that the mice previously referred to received more than twice these amounts of the drug.

Our limited experience with monkeys, as previously stated, merely served to indicate that under properly chosen conditions, they too would respond to repeated doses in essentially the same way as rabbits or mice. While we do not know what the lethal dose of this drug is for monkeys, it is probably not less than 1 to 1.25 gm. per kilo of body weight, or approximately the same as that for guinea pigs and considerably more than that for the rabbit. In the one instance in which gradually increasing doses of A 63 were given to a monkey, we succeeded in giving as much as 1.5 gm. per kilo as the final dose with very slight intoxication resulting. When this animal was killed 3 months later for pathological examination, the organs appeared normal.

CONCLUSION.

The essential facts to be gathered from these studies of the toxicologic action of *N*-phenylglycineamide-*p*-arsonic acid may be summarized very briefly. The substance is one which lends itself well to almost any method of administration and can be given to animals in very large doses. The tolerance of different animal species varies rather widely but with one exception the reaction of laboratory animals to toxic doses of the drug is of favorable character. That is, toxic effects are confined to doses relatively close to the minimum lethal dose and the recovery of animals from sublethal intoxications is remarkably rapid and complete. This feature of the action of the drug makes possible the repeated administration of even very large doses at comparatively short intervals of time without incurring the dangers incident to cumulative action or to superposition of toxic effects. On the contrary, by taking advantage of this peculiarity of action, it is possible to develop such a degree of tolerance on the part of animals that the dose of the drug administered can be progressively

increased to a point well above that which is fatal to the normal animal, and this stands out as the feature of the toxicologic action of *N*-phenylglycineamide-*p*-arsonic acid which is of greatest significance in the use of the drug for therapeutic purposes.

CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

BIOLOGICAL SERIES. II.

THE THERAPEUTIC ACTION OF N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID IN EXPERIMENTAL TRYPANOSOMIASIS OF MICE, RATS, AND GUINEA PIGS.

BY LOUISE PEARCE, M.D., AND WADE H. BROWN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 18, 1919.)

The arsenical compound *N*-phenylglycineamide-*p*-arsonic acid, the toxic action of which has been described in the preceding paper, exercises a powerful therapeutic effect in experimental trypanosomiasis of laboratory animals. Trypanosomiasis as it occurs in mice and rats is chiefly characterized by the constant and progressively increasing number of trypanosomes in the peripheral blood stream, by the usual lack of any clinical manifestations, and by the relatively early death of the infected animal. An effective therapeutic compound, therefore, for the treatment of trypanosomiasis of mice and rats must be biologically available within a very short time after its administration and must have a sufficient speed and duration of action to halt and eventually overcome a rapidly increasing blood infection comparable, in part, with a bacteriemia, which if not checked, will cause the death of the animal in a few days or even hours. On the other hand, trypanosomiasis of many of the larger animals, especially of the rabbit, is preeminently a tissue infection and is characterized, in the acute stages, by conspicuous edematous and inflammatory swellings of the soft parts, particularly of the head and external genitalia, together with loss of appetite, weakness, and emaciation, while in the more chronic phases of the infection, the inflammatory lesions undergo induration and even necrosis with involvement of the deeper tissues including the periosteum and bone. The duration of the infection in

rabbits is a matter of weeks or months and the presence or absence of trypanosomes in the blood stream is distinctly of minor importance. It is obvious that a drug which is used for the treatment of this type of infection must possess in addition to trypanocidal action, the power of penetrating diseased tissue, and since this may require a considerable amount of time, the drug in question must remain biologically active in the animal host as long as may be necessary.

In order, therefore, to arrive at a full appreciation of the therapeutic action of a drug in experimental trypanosomiasis, one has, from the point of view of the animal factor, two general types of infection, the treatment of which will furnish information as to the speed of action on the one hand and as to the duration of action or potency of the drug on the other. This information, which should include such data as the determination of a therapeutic range in different animal species as well as curative doses, the therapeutic action against different species of trypanosomes, the comparative value of different routes of administration, and the results of a repeated dose system of therapy, together with the data upon the toxicological and pathological action of the drug in question in various animal species, furnishes a logical foundation for an accurate appraisal of the action of the drug under experimental laboratory conditions and, at the same time, furnishes a basis for estimating its probable value in the treatment of trypanosomal infections as they occur in nature.

The present paper includes the therapeutic results obtained with the amide of *N*-phenylglycine-*p*-arsonic acid in experimental trypanosomiasis of mice, rats, and guinea pigs. While trypanosomiasis of guinea pigs is, strictly speaking, a chronic infection of cyclic character, the results of its treatment with this drug are incorporated with those of acute infections of mice and rats.

EXPERIMENTAL.

Strains of *Tr. brucei*, *Tr. gambiense*, *Tr. equinum*, *Tr. equiperdum*, and *Tr. evansi*, carried constantly in stock mice and stock guinea pigs, were used for the infecting organisms, and all inoculations were made intraperitoneally. The details of animal inoculation for therapeutic purposes and the general conduct of such experiments have been de-

scribed¹ and need not be repeated here; but in this connection, mention may be made of the desirability of regulating and maintaining a uniform grade of infection, a factor of importance in interpreting therapeutic results. In the majority of experiments, we used a strain of *Tr. brucei* which is highly virulent and uniformly fatal for mice, rats, and guinea pigs, mice dying in from 60 to 70 hours, rats in from 4 to 5 days, and guinea pigs on an average of 4 weeks after the usual intraperitoneal inoculation. With organisms of less virulence, the size of the inoculating dose was proportionately adjusted to insure an infection comparable with that produced by our strain of *Tr. brucei*.² Both mice and rats were treated 18 to 24 hours and guinea pigs usually 1 week after inoculation, after parasites had been demonstrated in the peripheral blood. All the untreated controls died irrespective of the species of trypanosome used. Daily examinations of the blood were made in the mouse experiments for the 1st week or 10 days and at frequent intervals thereafter, while with rats and guinea pigs, the examinations usually began with 2 day intervals. The treated animals were kept under observation from 1 to 3 months.

Doses of the drug used are expressed in grams of the sodium salt per kilo of body weight. The solutions were always freshly prepared with sterile distilled water in various concentrations (0.5, 1, and 2 per cent) and the intraperitoneal route of administration was usually employed. The concentration of the drug solution in any given experiment remained a constant, the volume of the dose being the variable quantity.

Treatment of Experimental Trypanosomiasis of Mice.

For convenience of comparison, we have summarized, in the accompanying tables, the various protocols of individual experiments which were undertaken for the purpose of ascertaining the range of therapeutic action of the drug. We have included under the heading "intercurrent deaths" those animals which died from accidental

¹ Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1918, xxviii, 109.

² In the case of *Tr. equinum*, the infection produced in several mouse and rabbit experiments was of an unusually severe nature, a condition which may be referable to an increase in the virulence of the strain at the time of inoculation or to the strength of the inoculating dose employed.

causes within the first 15 days after treatment in the cases of *Tr. brucei*, *Tr. gambiense*, and *Tr. equinum*, and within the first 30 days in the cases of *Tr. equiperdum* and *Tr. evansi*, since it was found that relapses practically always occurred within these periods of time with our strains of these species of trypanosomes, and none of the animals listed under "intercurrent deaths" is included in the number of cured animals irrespective of the size of the dose administered.

Trypanosoma brucei.—Table I shows the results of the treatment of *Tr. brucei* infection in 75 mice with graded doses of the amide of

TABLE I.

Tr. brucei Infection of Mice Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
1.0	2	0	0	2
0.9	2	0	0	2
0.75	2	0	0	2
0.6	3	0	0	3
0.5	1	0	0	1
0.4	2	1	0	1
0.35	1	0	0	1
0.3	11	1	0	10
0.275	5	0	0	5
0.25	19	1	2	16
0.225	13	0	3	10
0.2	13	0	8	5
0.15	1	0	1	0
Total.....	75			

N-phenylglycine-*p*-arsonic acid ranging from 0.15 to 1 gm. per kilo of body weight administered intraperitoneally. The majority of animals were treated with doses of from 0.2 to 0.3 gm., since doses above this level seemed uniformly curative, and in fact, no relapse was demonstrable with amounts of 0.275 gm. or more. Two mice, one treated with 0.4 gm. and the other with 0.3 gm., died from accidental causes within the first 15 days and consequently were not included in the number of cured animals, although in neither case could trypano-

somes be demonstrated. All the other mice which were treated with doses ranging from 0.275 to 1 gm. showed no relapse and all lived 30 days after treatment. Doses of 0.2, 0.225, and 0.25 gm. per kilo of body weight were found to exert a definite therapeutic action, although at these levels relapses occurred. With 0.25 gm., two out of nineteen mice relapsed and one died from intercurrent causes; with 0.225 gm., three relapses occurred out of thirteen mice; with 0.2 gm., there were eight relapses out of thirteen treated animals. With doses at this level, relapses usually appeared from 3 to 9 days after treatment. In some instances with doses of 0.2 and 0.225 gm., parasites were never completely cleared from the blood but they were definitely less numerous than in the blood of the untreated controls, and in addition, these mice lived longer than the controls.

TABLE II.

Tr. brucei Infection of Mice Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Intravenous Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.6	1	0	0	1
0.5	1	0	0	1
0.4	1	0	0	1
0.3	1	0	0	1
0.25	2	0	0	2
0.2	1	0	0	1
0.15	1	0	1	0
0.1	1	0	1	0
0.05	1	0	1	0
0.025	1	0	1	0
Total.....	11			

The number of mice treated intravenously and subcutaneously, shown in Tables II and III, is relatively small, yet the therapeutic results by such routes are in striking accord with those obtained by intraperitoneal administration of the drug. Of eleven mice treated intravenously with doses ranging from 0.025 to 0.6 gm. per kilo, four animals which were given doses of 0.025, 0.05, 0.1, and 0.15 gm. respectively were not cured and the blood of those receiving the three lowest doses was never cleared of parasites, while the mouse

which was treated with 0.15 gm. relapsed on the 4th day. Doses of 0.2 gm. and above were uniformly curative as contrasted with the slightly higher level of 0.275 gm. by intraperitoneal administration.

Fourteen mice were treated subcutaneously, as indicated in Table III, with doses of 0.2, 0.25, and 0.3 gm. with no relapses, although there was one intercurrent death in the first fortnight which is not

TABLE III.

Tr. brucei Infection of Mice Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Subcutaneous Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.3	4	0	0	4
0.25	5	1	0	4
0.2	5	0	0	5
Total.....	14			

included in the number of cures. From these results, it would seem that both intravenous and subcutaneous routes of administration of the drug in *Tr. brucei* infections of mice were equally efficacious, since in both the dose of 0.2 gm. per kilo of body weight was uniformly curative, a dose which represents the lower limit of therapeutic action by intraperitoneal administration as contrasted with the curative dose of 0.275 gm. by this route.

Trypanosoma gambiense.—46 mice infected with *Tr. gambiense* were treated intraperitoneally with doses of the drug ranging from 0.125 to 0.3 gm. per kilo of body weight (Table IV). There were four demonstrable relapses; one mouse treated with 0.25 gm. relapsed on the 11th day, one with 0.15 gm. also on the 11th day, and two with 0.125 gm. on the 14th day. Of the fourteen mice which were given doses of 0.125 and 0.15 gm., three died within the first 15 days from accidental causes, three definitely relapsed, and eight animals survived the 30 day period of cure. Graded doses from 0.175 to 0.3 gm. were given to thirty-two mice, and of this number but one animal treated with 0.25 gm. relapsed. The range of therapeutic activity of the amide of *N*-phenylglycine-*p*-arsonic acid against *Tr. gambiense* infection in mice is definitely greater than against

Tr. brucei infections, since doses of 0.15 gm. failed to cure nagana mice and doses of 0.2 gm. were curative in but five out of thirteen animals.

TABLE IV.

Tr. gambiense Infection of Mice Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid
24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.3	2	1	0	1
0.275	2	0	0	2
0.25	8	0	1	7
0.225	4	1	0	3
0.2	9	0	0	9
0.175	7	1	0	6
0.15	9	2	1	6
0.125	5	1	2	2
Total.....	46			

Trypanosoma equinum.—The results of the treatment of *Tr. equinum* infection in mice are shown in Table V. Twenty-nine mice were treated with intraperitoneal doses of 0.05 to 0.25 gm. per kilo of body weight, with no relapses at 0.2 and 0.25 gm. Doses of 0.1 and 0.15 gm. had a distinct therapeutic effect, and seven of the four-

TABLE V.

Tr. equinum Infection of Mice Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid
24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.25	7	1*	0	6
0.2	7	3†	0	4
0.15	7	1	2	4
0.1	7	0	4	3
0.05	1	0	1	0
Total.....	29			

* Bacterial infection.

† Bacterial infection in one mouse.

teen mice treated with these doses were cured, while in some instances, the life of the uncured animal was considerably longer than that of the untreated control. A dose of 0.05 gm. had no effect whatever on the length of the infection or on the number of parasites in the peripheral blood in the single instance in which this dose was used.

Trypanosoma equiperdum.—In Table VI are given the results of the treatment of thirty-seven dourine mice with this arsenical compound in doses ranging from 0.15 to 0.5 gm. per kilo of body weight. The therapeutic action of the drug on infections in mice produced by our strain of *Tr. equiperdum* was quite irregular. Relapses

TABLE VI.

Tr. equiperdum Infection of Mice Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.5	4	0	3	1
0.45	4	1	2	1
0.4	4	0	3	1
0.35	6	0	5	1
0.325	2	1	1	0
0.3	6	1	4	1
0.275	2	0	1	1
0.25	4	0	2	2
0.225	2	0	1	1
0.2	2	0	2	0
0.15	1	0	1	0
Total.....	37			

occurred with all doses used irrespective of size, from 4 to 15 days after treatment, and there were no cures below doses of 0.225 gm., although in every instance, even with the lowest doses, the peripheral blood was cleared of trypanosomes for several days and the mice themselves lived from 2 to 8 days longer than the untreated controls. On the other hand, there were some cures at doses of 0.2 to 0.3 gm.

Trypanosoma evansi.—Thirty mice infected with *Tr. evansi* were treated intraperitoneally with the amide of *N*-phenylglycine-*p*-arsonic acid in doses ranging from 0.15 to 0.75 gm. (Table VII).

There were definite cures at all doses above 0.15 gm. with no demonstrable relapse above 0.5 gm. Of the four mice treated with 0.75 gm., one died within the first 30 days after treatment and three were cured. With doses ranging downward from 0.5 gm., relapses occurred irregularly in from 7 to 15 days, but the trypanocidal activity of the drug was shown in all instances even with the lowest doses, since the blood was cleared of parasites for several days following treatment.

TABLE VII.

Tr. evansi Infection of Mice Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.75	4	1	0	3
0.5	4	1	1	2
0.4	4	0	1	3
0.35	2	1	0	1
0.3	6	1	3	2
0.275	2	0	1	1
0.25	2	0	1	1
0.225	2	1	1	0
0.2	2	0	1	1
0.15	2	0	2	0
Total.....	30			

Treatment of Experimental Trypanosomiasis of Rats.

The therapeutic experiments with rats are likewise grouped in tables according to the various species of trypanosomes employed. As in the case of the mice experiments, the animals listed under "intercurrent deaths" are not included in the number of cured animals, irrespective of the time of occurrence of the death or of the size of the dose administered. Intraperitoneal administration of the drug was employed in every experiment.

Trypanosoma brucei.—Thirty-four rats infected with *Tr. brucei* were treated intraperitoneally with the amide of *N*-phenylglycine-*p*-arsonic acid in doses of from 0.2 to 1.25 gm. per kilo of body weight (Table VIII). There was but one demonstrable relapse at 0.225 gm.

in an animal which showed numerous trypanosomes in its blood 14 days after treatment. With doses of the drug as high as 0.75 gm., rats may show an early and severe intoxication from which recovery is unusual, and such was the case with the rat which was treated with 0.9 gm. and which died on the 4th day. Doses below 0.6 gm. are well borne and are uniformly curative in *Tr. brucei* infections down to 0.25 gm. However, with doses below this level, the therapeutic action of the drug is still marked as shown by the single relapse in five animals treated with 0.2 and 0.225 gm. doses.

TABLE VIII.

Tr. brucei Infection of Rats Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo. gm.	No. of rats.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
1.25	1	0	0	1
1.0	2	0	0	2
0.9	1	1	0	0
0.75	1	0	0	1
0.6	1	1	0	0
0.5	1	1	0	0
0.4	3	0	0	3
0.375	3	0	0	3
0.35	3	2	0	1
0.325	3	0	0	3
0.3	3	1*	0	2
0.275	3	2*	0	1
0.25	4	0	0	4
0.225	3	0	1	2
0.2	2	1	0	1
Total.....	34			

* Two pregnant females dying 7 and 8 days after treatment.

Trypanosoma gambiense.—Table IX shows the results of the treatment of twenty-five rats infected with *Tr. gambiense* with doses of from 0.1 to 0.3 gm. Doses of 0.2 gm. and above were uniformly curative, no relapses occurring in the fifteen animals treated with 0.2, 0.25, and 0.3 gm. per kilo of body weight. Ten animals were given 0.1 and 0.15 gm. doses, and of this number, there were three relapses and one accidental death. One rat which was treated with

0.15 gm. showed a well developed blood infection on the 18th day and died on the 25th day after treatment. The two rats which relapsed with doses of 0.1 gm. showed trypanosomes on the 10th and 18th days and died on the 25th and 36th days.

TABLE IX.

Tr. gambiense Infection of Rats Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of rats.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.3	5	0	0	5
0.25	5	2	0	3
0.2	5	2*	0	3
0.15	5	1	1	3
0.1	5	0	2	3
Total.....	25			

* Animals missing.

Trypanosoma equiperdum.—Eight rats infected with *Tr. equiperdum* were treated with the amide of *N*-phenylglycine-*p*-arsonic acid as shown in Table X. Doses from 0.25 to 0.5 gm. per kilo of body weight were used and no relapse occurred in the higher doses of 0.4 and 0.5 gm. Two rats treated with 0.3 gm. relapsed on the 8th and 16th days and one with 0.25 gm. relapsed on the 7th day. In each case, however, examination of the peripheral blood showed no parasites for several days and the animals all lived longer than the untreated controls.

TABLE X.

Tr. equiperdum Infection of Rats Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of rats.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.5	2	0	0	2
0.4	2	0	0	2
0.3	2	0	2	0
0.25	2	1	1	0
Total.....	8			

Trypanosoma evansi.—The results of the treatment of surra infection in rats are shown in Table XI. Nine rats were treated with doses ranging from 0.25 to 0.5 gm. per kilo of body weight, and no relapses were observed with doses above 0.25 gm. Of the three rats which were given 0.25 gm. of the drug, one relapsed with a heavy blood infection on the 14th day after treatment and died on the following day; a second animal which died on the 14th day showed at autopsy a large splenic tumor and was considered a probable relapse; while the third rat in this group, which died 13 days after treatment, is not counted as a cured animal, although no parasites were found in the peripheral blood and the autopsy findings were negative.

TABLE XI.

Tr. evansi Infection of Rats Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of rats.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.5	2	1*	0	1
0.4	2	0	0	2
0.3	2	0	0	2
0.25	3	1	2	0
Total.....	9			

* Pregnant female.

Treatment of Experimental Trypanosomiasis of Guinea Pigs.

A number of therapeutic experiments in guinea pigs have been done of which the example given in Table XII is typical. Doses of 0.15, 0.2, and 0.25 gm. per kilo of body weight of the compound given intraperitoneally in a 2 per cent solution were uniformly curative, no relapses having been detected during the period of observation of 3 months. The nagana infection in this particular experiment was severe, as shown by the death of the control animals on the 1st and 3rd days after treatment of the series. Other experiments in which the same and much larger doses of the drug were given showed uniformly curative results and need not be included here.

The therapeutic action of *N*-phenylglycineamide-*p*-arsonic acid upon experimental trypanosomiasis of guinea pigs is in striking accord with that obtained in mice and rats and in marked contrast to that of many trypanocidal drugs which show a greatly lessened therapeutic action in this animal species.

TABLE XII.

Tr. brucei Infection of Guinea Pigs Treated with *N*-Phenylglycineamide-*p*-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of guinea pigs.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
<i>gm.</i>				
0.25	2	1	0	1
0.2	2	0	0	2
0.15	2	0	0	2
Total.....	6			

DISCUSSION.

As has been shown in the accompanying tables, the curative level of the amide of *N*-phenylglycine-*p*-arsonic acid is quite constant and uniform for most species of trypanosome infections in both mice and rats. Certain exceptions exist which will be referred to later, but in general, with the more virulent strains of organisms and the grade of infection which we have used, single doses of from 0.2 to 0.3 gm. per kilo of body weight given intraperitoneally 24 hours after inoculation are uniformly curative. The parasites promptly disappear from the peripheral blood within 24 hours following treatment at this level of dosage and barring death from accidental causes, the animals survive the observation period of 1 or 2 months with no return of the infection. For mice the minimum lethal dose of this drug given intraperitoneally has been placed at 2 to 2.25 gm. per kilo of body weight, and since the curative dose may be considered as 0.275 gm., we have a curative ratio of 1:8. Similarly the minimum lethal dose of the drug for rats has been estimated at 0.75 gm. per kilo of body weight, and the curative ratio for rats, therefore, is 1:3.

In addition, the range of therapeutic activity, as indicated by the

dose that will temporarily clear the blood of trypanosomes as well as effect a certain proportion of cures, varies somewhat with the species of organism. Generally speaking, with the more virulent and more rapidly fatal trypanosomes, the clearing dose closely approaches the curative dose. Thus, with *Tr. brucei* infections in mice, 0.2, 0.225, and 0.25 gm. were usually clearing doses; while with *Tr. gambiense* and *Tr. equinum*, 0.1, 0.125, and 0.15 gm. cleared the blood. On the other hand, with less virulent strains, such as *Tr. equiperdum* and *Tr. evansi*, the clearing dose is considerably below the curative level. Thus, with these strains, doses of 0.15 to 0.25 gm. cleared the blood of trypanosomes for several days, although the curative dose for both strains must be placed above 0.5 gm. The range of therapeutic activity as expressed in the size of the clearing dose is practically the same in the rat as in the mouse infections.

Different species of trypanosomes vary considerably in their resistance to therapeutic agents, and in this respect, the therapeutic action of this compound is not maintained at the constant curative level of 0.2 to 0.3 gm. per kilo of body weight. Moreover, the animal species employed may to some extent influence the control of certain infections. The curative range of 0.2 to 0.3 gm. is quite constant for *Tr. brucei*, *Tr. gambiense*, and *Tr. equinum* infections in mice and *Tr. brucei* and *Tr. gambiense* infections in rats, but with *Tr. equiperdum* and *Tr. evansi* infections in mice, the dose required to effect a cure is considerably higher. The highest dose used, 0.5 gm., failed to cure all the mice infected with *Tr. equiperdum*, although there were cures at doses as low as 0.225 and 0.25 gm. With infections of *Tr. evansi*, mice treated with 0.75 gm. did not relapse, but below this level, cures were uncertain. Both these infections, however, were more easily handled in rats than in mice, since there were no relapses with doses of 0.4 gm. in *Tr. equiperdum* and none with 0.3 gm. in *Tr. evansi* infections.

The lower level of therapeutic action of the drug is quite sharp with the more virulent species of trypanosomes. Relapses with *Tr. brucei* infections in mice occurred with 0.25 gm. (intraperitoneal administration) and increased in number with lower doses, while with *Tr. gambiense* and *Tr. equinum* infections in mice, relapses began with 0.15 gm., with the single exception of one *Tr. gambiense* mouse which

relapsed with a 0.25 gm. dose. It is possible that this relapse might have been due to a leakage of the drug at the time of treatment, since this was the only observed relapse in twenty-eight mice treated with doses of from 0.175 to 0.25 gm. The majority of relapses with these infections occurred from 5 to 12 days after treatment. The level of relapse occurrence with *Tr. brucei* and *Tr. gambiense* infections in rats is quite comparable with that observed in mice, while the time of occurrence was from 14 to 25 days after treatment. With the less virulent organisms which were used, *Tr. equiperdum* and *Tr. evansi*, the therapeutic action of the amide of *N*-phenylglycine-*p*-arsonic acid is much less clear-cut and regular. Relapses occurred in mice with doses of from 0.15 to 0.5 gm. and in general from 7 to 15 days after treatment. The therapeutic action in rats, however, is more constant and more sharply limited, for with *Tr. equiperdum* infections, there were no relapses above 0.3 gm. and with *Tr. evansi* none above 0.25 gm. The relapses with both these species in rats occurred from 8 to 16 days after treatment. It will be recalled that in all the experiments with both mice and rats, and irrespective of the species of trypanosome employed, treatment was given on the day following the inoculation of the animals and that the therapeutic results described above apply to the treatment of a 24 hour infection. The average curative level, the clearing dose, and the number and time of relapses would undoubtedly be affected with any considerable change in the time of treatment.

The therapeutic results in the treatment of experimental trypanosomiasis of guinea pigs, as shown by the low curative dose of 0.15 gm. per kilo of body weight in a severe infection, are even better than those obtained in the treatment of mice and rats and are in striking contrast to the action of certain other trypanocidal agents. The curative ratio expressed in fractions of the minimum lethal dose is 1:10, since the lethal dose for guinea pigs is 1.5 gm. per kilo of body weight given intraperitoneally or subcutaneously.

The rapidity of the trypanocidal action of the amide of *N*-phenylglycine-*p*-arsonic acid on trypanosomiasis of mice, rats, and guinea pigs is quite marked. That is, with curative doses, the drug becomes quickly available after its administration and remains in an active biological state long enough to accomplish its trypanocidal action, for

in 18 to 24 hours after the treatment of a 24 hour infection, the peripheral blood is free of parasites. Moreover, with subcurative but clearing doses, a similar speed of trypanocidal action is shown. This speed of trypanocidal activity is operative in both mice and rats, but in the case of our strains of *Tr. equiperdum* and *Tr. evansi*, the action is apparently somewhat more drastic in rats. In mice the therapeutic action extends over a wide range of doses and cures are irregularly distributed among the low as well as among the high limits of this range, while treatment of the same infections in rats reveals a more sharply limited therapeutic action. It is of interest in this connection to compare the results of the administration of the drug by different routes in the treatment of *Tr. brucei* infections of mice. By all three routes, intraperitoneal, intravenous, and subcutaneous, the sharpness of therapeutic action is maintained within the curative range and apparently the drug is as available in an active biological state by subcutaneous as by intraperitoneal or intravenous administration. The therapeutic experiments in guinea pigs are too few to admit final analysis, but apparently the rapidity and sharpness of action of the drug likewise obtain in this animal species.

One other point regarding the action of the drug should be mentioned and this is in connection with the general condition of the treated animals. Practically without exception, all the cured animals showed a marked improvement manifested by a gain in weight and by the healthy appearance of their coats which dated from the time of treatment and continued during the period of observation. The cured animals were usually in a better condition than those of the original stock. Rats which were treated with high doses approaching a lethal level and which developed toxic manifestations did not recover, but aside from this evidence of drug toxicity, the treated animals were uniformly in excellent state. The negative autopsy findings in animals that were treated with therapeutic doses of the amide of *N*-phenylglycine-*p*-arsonic acid furnish additional corroborative evidence of the non-injurious action of such doses to the host.

CONCLUSIONS.

N-Phenylglycineamide-*p*-arsonic acid is an agent of marked therapeutic action in the treatment of experimental trypanosomiasis of mice, rats, and guinea pigs. It possesses an average curative range of from 0.2 to 0.3 gm. per kilo of body weight of the sodium salt against a 24 hour infection in mice and rats produced by several species of pathogenic trypanosomes. Since the lethal dose for mice is from 2 to 2.25 gm. and for rats 0.75 gm. per kilo of body weight, we have curative ratios of 1:8 and 1:3 respectively. The curative dose for guinea pigs is 0.15 gm. per kilo of body weight, thus giving a curative ratio of 1:10. The trypanocidal activity of this compound is relatively rapid in all three animal species, for the peripheral blood is cleared of organisms within 24 hours after its administration, and in addition, the lower limits of the curative range are comparatively sharply defined. Intraperitoneal, intravenous, and subcutaneous routes of administration for all practical purposes may be considered equally efficacious in *Tr. brucei* infections of mice both as regards the speed of action of the drug and the average curative range. The administration of the drug in therapeutic amounts in all three animal species is not followed by manifestations of organic or functional injury, but, on the contrary, the general physical condition of the treated animals shows an immediate and continued marked improvement.

The therapeutic activity in trypanosomiasis of mice, rats, and guinea pigs as evidenced by the relative speed and sharpness of action, together with the curative ratio as expressed in fractions of the minimum lethal dose, and the absence of organic injury or functional disturbance following therapeutic doses are significant and characteristic features of the amide of *N*-phenylglycine-*p*-arsonic acid.

CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

BIOLOGICAL SERIES. III.

THE THERAPEUTIC ACTION OF N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID IN EXPERIMENTAL TRYPANOSOMIASIS OF RABBITS.

BY LOUISE PEARCE, M.D., AND WADE H. BROWN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 17 TO 37.

(Received for publication, June 18, 1919.)

Experimental trypanosomiasis of rabbits, as has been pointed out, presents many points of similarity with naturally acquired trypanosomiasis of human beings and animals, and since the treatment offers an opportunity of obtaining fundamental information which may be of practical importance in the treatment of trypanosomal disease as it exists in nature, we have endeavored to emphasize such fundamental points in the description of the therapeutic results with the amide of *N*-phenylglycine-*p*-arsonic acid reported in this paper.

EXPERIMENTAL.

Condition of Experiments.—The majority of therapeutic experiments were carried out with strains of *Tr. brucei* and *Tr. gambiense* and a smaller number of experiments with *Tr. equiperdum*, *Tr. equinum*, and *Tr. evansi*. The infection produced by all five strains was uniformly fatal in untreated rabbits. The infecting virus in all experiments was, with one exception, obtained from stock guinea pigs in which the trypanosome strains were continuously carried, thus conforming to the principle of the influence of the animal species upon the type of infection which has been discussed in a previous paper.¹ In addition,

¹ Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1918, xxviii, 109.

in order to secure regularity of incubation as well as uniformity of character and termination of the infection in as far as this was possible, blood was taken from stock guinea pigs which showed a well developed infection and the inoculating doses of the blood suspension were given intravenously. With *Tr. brucei*, *Tr. equiperdum*, and *Tr. evansi*, 1 cc. per kilo of body weight of a + suspension was inoculated, while with *Tr. gambiense* and *Tr. equinum*, 1 cc. per kilo of a ++ to +++ suspension was used. Under these conditions of experimental procedure, rabbits infected with *Tr. brucei* showed initial signs of the disease in from 5 to 7 days and the untreated animals survived from 1 to 3 months, although the majority of controls died within the first 5 weeks, while the first signs of the infection with *Tr. gambiense* were noted 7 to 15 days after inoculation and untreated animals survived on an average of 8 weeks. The clinical course of the infection produced in rabbits by our strains of *Tr. equinum*, *Tr. evansi*, and *Tr. equiperdum* is, on the whole, comparable with that caused by *Tr. brucei*.

Rabbits thus infected were treated with the sodium salt of the amide of *N*-phenylglycine-*p*-arsonic acid at a time when the infection was well established, usually about 10 to 15 days after inoculation with *Tr. brucei* and at a somewhat longer period of time with the other strains of trypanosomes, when well marked clinical signs of the disease together with loss of appetite, weakness, and a concomitant loss of weight or even emaciation were present. In a certain number of cases, particularly in the treatment of relapses, rabbits showing a more advanced or even a chronic stage of trypanosomiasis were used.

With regard to the matter of dosage, it is important to recognize that since individual rabbits vary considerably in their reaction to trypanosomal infections as shown by the character and degree of the clinical signs, we have endeavored to utilize this factor by placing some of the animals most affected at the crucial dose levels, that is, within the curative range. The dose of the drug for each animal was calculated in grams per kilo of body weight, weighed on a balance, dissolved in sterile distilled water, and injected by a syringe into the marginal ear vein except in the few instances in which the drug was administered intramuscularly, subcutaneously, or *per os* through a stomach tube. The concentration of the drug solutions varied from 5 to 50 per cent, although in the majority of experiments a 5 per cent solution was used.

The immediate and ultimate therapeutic effect of the drug in the treatment of experimental trypanosomiasis in rabbits was closely observed throughout a period of at least 3 months and in many instances for a longer period of time, although the majority of relapses after non-curative doses of the amide of *N*-phenylglycine-*p*-arsonic acid occurred in our experiments within 2 to 5 weeks after treatment.

Determination of a Single Dose Curative Range.

Our initial experiments with the amide of *N*-phenylglycine-*p*-arsonic acid were largely concerned with the determination of the range of therapeutic action and a study of the effect of the drug within this range rather than the determination of a single curative dose or an attempt to ascertain the ideal method of treating and of curing experimental trypanosomiasis. Although the treatment of trypanosomiasis in this animal species resolves itself for the most part into the treatment of individuals, we have grouped together in Table I the results of a number of experiments which have certain features in common. In these experiments, thirty-four rabbits infected with *Tr. brucei* were treated 8 to 15 days after inoculation, at a time when all showed well marked clinical signs and symptoms of the disease. Some of the control animals died from 20 to 36 days after treatment, while others which showed extremely advanced signs at the end of 42 days were used for therapeutic purposes. The single doses of the drug employed in the treatment of these rabbits, as is shown in the table, ranged from 0.75 to 0.1 gm. per kilo of body weight and were administered intravenously in a 5 per cent solution.

There were no evidences of drug intoxication after treatment with single doses of this range and the clinical signs of trypanosomiasis were quickly influenced. Usually the acute manifestations of the infection, such as edema, capillary dilatation and congestion, and swelling of the base of the ears, eyelids, prepuce, and testicles, largely subsided within 24 to 48 hours, while the reduction of the more indurated and advanced lesions required from 2 to 4 days. The immediate clinical results of the treatment are illustrated in Figs. 1 to 7. Apart from the outward signs of the disease, the animal's general condition improved markedly following the injection of the drug as shown by

a rapid return of appetite and an increase in weight, so that within a few days emaciation had given place to a more or less well nourished condition, while in the succeeding weeks, a normal increase in weight occurred.

The final results in this series are given in Table I. There were no relapses among the twelve rabbits treated with doses of both 0.35 and 0.25 gm. per kilo of body weight, while with the intermediate dose of

TABLE I.

Results Obtained in the Treatment of Rabbits Infected with Tr. brucei with Single Doses of N-Phenylglycineamide-p-Arsonic Acid, Administered Intravenously.

Dose per kilo.	No. of rabbits.	No. of intercurrent deaths.	No. of relapses.	No. of cures.	Percentage of cures.
<i>gm.</i>					
0.75	1	0	0	1	100
0.4	2	0	0	2	100
0.35	6	2*	0	6	100
0.3	7	0	1	6	86
0.25	6	3†	0	6	100
0.2	10	1‡	3	7	70
0.1	2	0	2	0	0
Total.....	34				

* One was killed 71 days after treatment; extensive ringworm. One died 73 days after treatment; middle ear abscess.

† One died the day before the series was discarded; hemorrhagic septicemia. One died 71 days after treatment; acute pneumonia. One was killed 71 days after treatment; extensive ringworm.

‡ Killed 77 days after treatment; bacterial infection.

0.3 gm. per kilo, there was one relapse out of seven animals, or 86 per cent of cures. Furthermore, with a lower dose of 0.2 gm. seven out of ten rabbits were cured (70 per cent), and finally, the two rabbits treated with the very low dose of 0.1 gm. relapsed, although the marked therapeutic activity of the drug at this level is shown by the fact that with rabbits so treated, regression and healing of the lesions took place very rapidly and the relapses did not occur until 11 and 21 days after treatment. There were six incidental deaths among the thirty-four rabbits included in this table, but since they occurred over 10 weeks after treatment and were in no way associated with evidences

of trypanosomiasis, we have not excluded them from the number of cured animals.

The outstanding feature of the treatment of an acute, progressive, and well established *Tr. brucei* infection in rabbits with single doses of the amide of *N*-phenylglycine-*p*-arsonic acid as shown in Table I is a well marked sharply limited curative range of therapeutic action with doses varying from 0.2 to 0.35 gm. per kilo, and that within these limits, the percentage of cures is, on the average, high. Thus, there were twenty-nine rabbits treated with doses of 0.2, 0.25, 0.3, and 0.35 gm. per kilo of body weight and of these four relapsed and twenty-five, or 86.2 per cent, were cured. In addition, both upper and lower limits of action of the curative range are sharply marked—with doses of 0.2 gm., there were 70 per cent of cures and with the lower dose of 0.1 gm., there were no cures although this amount of the drug possessed a marked therapeutic effect, while, on the other hand, doses of 0.35 gm. or higher were uniformly curative. If we consider that the minimum lethal dose of the drug for rabbits is from 0.75 to 0.9 gm. per kilo of body weight when given intravenously and that the curative range of single doses is from 0.25 to 0.35 gm., we have a curative ratio of from one-third to one-half as expressed in fractions of the minimum lethal dose.

Much the same range of curative action is operative in rabbit trypanosomiasis produced by *Tr. gambiense* as is shown in Table II. In this series, eighteen rabbits were treated intravenously with doses of the amide of *N*-phenylglycine-*p*-arsonic acid of 0.35 to 0.15 gm. per kilo of body weight. There was a very noticeable regression of the signs of the infection on the 1st and 2nd days after treatment, and no detectable signs remaining on the 5th day, as may be seen in Figs. 8 and 9. Two out of the three rabbits treated with 0.15 gm. per kilo relapsed 20 and 33 days after treatment, and there was one relapse at 0.25 gm. 26 days after treatment, but the eleven rabbits treated with the other three doses of 0.2, 0.3, and 0.35 gm. per kilo were cured. Thus the curative range of the compound in *Tr. gambiense* infections of rabbits may be considered to be from 0.2 to 0.3 gm. per kilo when administered as single doses intravenously—or from one-fourth to one-third of the minimal lethal dose.

Our experience with the effect of this compound upon rabbit trypanosomiasis produced by other species of trypanosomes is limited

so that we are unable to draw final conclusions as to the ultimate curative range of the drug in these particular infections. At the time the experiments were done, the infection produced in these animals by our strain of *Tr. equinum* was unusually severe as was also the case in certain mouse experiments, and in addition, the upper range of the doses used was apparently too low. However, single doses as small as 0.2 gm. per kilo of body weight caused a complete disappearance of the acute signs of the disease in 3 days, although relapses eventually occurred with the doses 0.15, 0.2, 0.25, and 0.3 gm. per kilo. It was obvious that larger doses should have been used or a different system of treatment followed to effect a permanent cure in an infection as

TABLE II.

Results Obtained in the Treatment of Rabbits Infected with Tr. gambiense with Single Doses of N-Phenylglycineamide-p-Arsonic Acid, Administered Intravenously.

Dose per kilo.	No. of rabbits.	No. of intercurrent deaths.	No. of relapses.	No. of cures.	Percentage of cures.
<i>gm.</i>					
0.35	3	0	0	3	100
0.3	4	0	0	4	100
0.25	3	1*	1	1	50
0.2	5	1†	0	4	100
0.15	3	2‡	2‡	1	33½
Total	18				

* Died 6 days after treatment; bacterial infection.

† Died 20 days after treatment; gastroenteritis.

‡ Relapse plus bacterial infection 20 and 33 days after treatment.

severe as was the case in this particular experiment, since the control rabbit died the day after the animals in the series were treated and relapses occurred with such promptness and severity.

Two rabbits heavily infected with *Tr. equiperdum* which were treated with single doses of 0.5 and 0.3 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid per kilo of body weight, 36 days after inoculation, at a time when the disease was advanced, showed a prompt recovery from the clinical signs as is illustrated by Figs. 12 and 13. Both rabbits subsequently relapsed on the 47th and 57th days after treatment but were successfully retreated with repeated doses of the drug with no ultimate recurrence of the infection.

The therapeutic effect of the compound upon *Tr. evansi* in rabbits was studied in but one animal. The infection was well established on the day of treatment, 36 days after inoculation, as is shown in Fig. 14, when 0.3 gm. per kilo was administered intravenously. There was very little change in the clinical signs during the first 48 hours but on the following days, the improvement was marked (Figs. 15 and 16). Thick crusts about the nose and upper lips had shelled off in 10 days time and by the end of the 3rd week, the skin in these areas was healthy in appearance and the hair had begun to return (Fig. 17). There was no relapse and the rabbit was in excellent condition when it was discarded 113 days after treatment.

Summing up the results of the treatment of trypanosomiasis with single doses of the drug, we may say that with infections of average severity and duration produced by *Tr. brucei* and *Tr. gambiense*, the drug exercises a prompt and lasting therapeutic action in doses of from 0.2 to 0.35 gm. per kilo of body weight, and, furthermore, a marked therapeutic effect of some duration in doses of 0.1 and 0.15 gm. per kilo of body weight. Although the experimental evidence with regard to *Tr. evansi* infection in rabbits is insufficient for final conclusions, it is probable that the same range of curative action would apply to such rabbit infections as are produced by the particular strain of surra with which we have worked. On the other hand, single doses of the compound which fall within this curative range failed to cure *Tr. equinum* and *Tr. equiperdum* infections in rabbits. However, it should be pointed out that in both these experiments, especially with *Tr. equinum*, the disease was extremely marked and more advanced at the time of treatment than was the case with the other three infections, and it is possible that with more comparable experimental conditions, a certain proportion of cures could be obtained with single doses falling within the curative range of 0.2 to 0.35 gm. per kilo of body weight.

In addition to the above experiments, a series of twelve rabbits with a very severe *Tr. brucei* infection of 23 days duration was treated intravenously with large single doses in order to ascertain the reaction of extremely ill animals to the drug as compared with that of normal animals. The dose of 0.9 gm. per kilo of body weight caused toxic symptoms in the three rabbits so treated, with death on the 2nd and

3rd days, while a similar result followed the dose of 0.75 gm. in two out of three rabbits; with 0.6 gm., only one rabbit of the three became toxic and eventually recovered, and the dose of 0.5 gm. caused no toxic disturbances whatever. From the above results, it is evident that the tolerance of very ill rabbits suffering from a severe acute trypanosomiasis infection compares very favorably with that of normal animals to single large doses of *N*-phenylglycineamide-*p*-arsonic acid administered intravenously.

Treatment of Initial Infections with Repeated Doses.

In many respects, the treatment of rabbit trypanosomiasis with repeated small doses of the amide of *N*-phenylglycine-*p*-arsonic acid more nearly meets conditions as they are found in nature, and in addition the control and ultimate cure of severe and long standing infections which might not yield to a single dose treatment may often be successfully accomplished by this system of therapeutic procedure. This is due in part to the fact that with the debilitated and weak condition of the animal host, it may not be wise to give a single dose of sufficient size and probably in part also to the chronic and indurated nature of the lesions present, the penetration of which may be difficult and may require considerable time. By observing the action of single small doses of the drug, we form an idea of their effect and duration. Obviously, the repetition of a dose should occur before the effect of the previous dose has ceased, and we consider the proper spacing of doses as important a factor in this connection as the number and size of the doses. Finally, all these factors must be largely determined by the physical state of the diseased animal as well as by the age, severity, and type of the infection.

We have treated a number of rabbits infected with *Tr. brucei* with three doses of the compound ranging from 0.2 to 0.05 gm. per kilo repeated at various intervals of time, of which the following are typical examples. Two rabbits infected with *Tr. brucei* were treated 11 days after inoculation, with three doses of 0.2 gm. per kilo repeated at 2 day intervals. The disease was well established with marked typical clinical signs at the time of treatment. Both animals recovered promptly with no signs of drug intoxication and no return of the

infection. Two other rabbits in the same series treated with three doses of 0.15 gm. at 2 day intervals likewise showed a prompt and complete disappearance of the clinical signs of the infection but one animal eventually relapsed 30 days later. Another animal which was given three doses of 0.1 gm. at 2 day intervals remained free from any signs of the infection for 186 days at which time it was discarded as cured. The last rabbit in this series which was given three doses of 0.05 gm. relapsed 13 days after treatment had been begun.

A modification of the spacing of repeated small doses was followed in the case of another animal which was treated 14 days after inoculation with *Tr. brucei*; the initial dose was 0.1 gm. per kilo. 8 days after treatment, the return of clinical signs of trypanosomiasis was observed about the head and nose, and the dose of 0.1 gm. per kilo was repeated 2 days later. The clinical signs cleared up promptly within 4 days and the animal remained in good condition without relapse during the period of observation following treatment.

In the experiments just cited, the infections were of an acute, rapidly progressive type. The effect of treatment with repeated doses of this arsenical upon a slowly developing severe infection of long standing is illustrated in Figs. 18 to 23. In this instance, the rabbit was inoculated with *Tr. brucei* in the usual manner but the infection was relatively slow in developing and treatment was not begun until 48 days after inoculation, when three doses of 0.2 gm. were given intravenously on succeeding days. At this time, the animal was extremely weak and emaciated, the ears, eyelids, face, nose, and external genitalia were swollen and indurated, and in addition, there were a mucopurulent exudate and scab formation about the eyes and nose (Fig. 18). An immediate improvement in the clinical manifestations of the disease was seen after treatment, although there was a loss of weight for the first 5 days, but by the 7th day this had largely been regained. Within the week following treatment, the scabs about the ears and around the eyes and end of the nose and lips had scaled off almost entirely, leaving a fairly healthy looking skin, the indurated swellings about the head and genitalia had greatly subsided, and the general condition of the rabbit had improved remarkably as is shown in Figs. 19 and 20. By the end of 3 weeks, the change in the appearance of the rabbit as contrasted with its condition on the day of treatment was

quite striking (Fig. 22), and in 6 weeks, a small bald patch at the base of the left ear was the only residual sign of a previous lesion (Fig. 23). The recovery from a severe and long standing infection under the system of three small doses of 0.2 gm. per kilo repeated at daily intervals was complete and lasting, for there was no subsequent relapse during the period of observation of 195 days.

The treatment of initial infection with *Tr. brucei* in rabbits, therefore, may be successfully accomplished by a system of repeated small doses provided that the repetitions occur well within the time of duration of action of the previous dose. The value of this system of therapy is especially marked in instances of greatly weakened animals or in those in which chronic indurated lesions are pronounced.

Treatment of Relapses with Single Doses.

The successful treatment of trypanosomiasis relapses in rabbits, many of which were intentionally produced as test objects, is based largely upon the severity and duration of the relapse and the general physical condition of the rabbit. While the factor of fastness of trypanosomes due to previous treatment unquestionably plays a part in the blood infections of mice and rats and must be considered to a certain extent in the tissue infection of rabbits, it is our opinion that the severity and duration of the relapse as manifested by the signs and symptoms of the actual infection, together with the general physical state of the rabbit host, play a more important rôle. It is obvious that the treatment of a relapse is almost wholly the treatment of individuals, and hence no rules of procedure can be laid down; but because of the inherent condition imposed by the infection, we believe that in most cases relapses can best be handled by a system of repeated doses, the size and time of repetition of which are necessarily determined by the extent, severity, and age of the disease together with the physical condition of the animal host. However, if a single large dose is used, it must of necessity cause no serious degree of intoxication or pathological injury, and yet at the same time, it must be of sufficient strength to penetrate diseased tissues of more or less induration and in like manner to act as a trypanocidal agent for a sufficient length of time. A number of relapses in rabbits have

been treated with single doses of this arsenical, of which the following will serve as examples.

The appearance of a *Tr. brucei* relapse of a week's duration at the time of retreatment is shown in Fig. 24. This rabbit was originally treated intravenously with three doses of 0.05 gm. per kilo of the drug which had served to clear up all outward manifestations of the disease, but 13 days later, definite signs of a relapse were noted. The infection was allowed to progress for a week at which time the clinical signs had rapidly increased both in degree and extent. The general physical condition of the rabbit was good. The entire left side of the face was greatly swollen and the left eye completely closed; the upper eyelid was very puffy, and protruding and escaping from between the margins of both lids, which were reddened with considerable loss of hair, was a slightly purulent exudate. The right upper eyelid, right cheek, and upper lip were also slightly swollen. Both ears, especially the left, which drooped markedly, were swollen, inflamed, and edematous and the right testicle was somewhat indurated. The dose selected for retreatment in this instance was 0.5 gm. per kilo of the amide of *N*-phenylglycine-*p*-arsonic acid given intravenously; it was followed by no toxic symptoms whatever. There was a most marked reduction of the clinical signs on the 2nd day; in a week's time the appearance of the rabbit was quite normal as is shown in Fig. 25, and this continued with no return of the infection during the period of observation of 166 days.

Another nagana rabbit which had been treated intravenously with the single dose of 0.29 gm. of the drug per kilo of body weight relapsed 26 days after treatment and the signs of the disease increased extremely rapidly for the succeeding 8 days. A single dose of 0.4 gm. per kilo of the drug was given intravenously. This was followed by a marked immediate improvement in the clinical signs of the infection and in 10 days the animal had regained its original weight and its appearance was entirely normal. There was no further relapse during the 149 day period of observation.

In addition to the treatment of rabbits infected with *Tr. brucei* which had relapsed after the initial treatment with the amide of *N*-phenylglycine-*p*-arsonic acid, a number of animals were treated that had relapsed from initial treatment with single doses of arsphenamine

and neoarsphenamine (German salvarsan and neosalvarsan), ranging from 0.005 to 0.02 gm. per kilo of body weight. For purposes of comparison, the original series which included these rabbits was conducted under the same conditions as were the parallel experiments in which the amide of *N*-phenylglycine-*p*-arsonic acid was used, and the infection was of the same acute nature with marked clinical signs at the time of treatment. The animals which relapsed after the treatment with arsphenamine and neoarsphenamine were held until the infection had progressed to an extreme degree with conspicuous clinical signs and a generally poor physical state of the animals; they were then treated with single doses of *N*-phenylglycineamide-*p*-arsonic acid. Five animals were given 0.5 and eight were given 0.4 gm. per kilo of body weight and all promptly recovered from the signs of the infection, with no evidences of drug intoxication and with a very noticeable and rapid improvement in their physical condition. Three rabbits in this group were killed 34, 47, and 56 days after treatment because of extensive ringworm or middle ear disease, but at autopsy no evidence of trypanosomiasis was found and none of the remaining ten rabbits suffered a relapse from the retreatment with *N*-phenylglycineamide-*p*-arsonic acid.

From the experimental evidence, therefore, it is seen that rabbits infected with our strain of *Tr. brucei* which have relapsed with severe clinical signs after insufficient treatment with the amide of *N*-phenylglycine-*p*-arsonic acid, arsphenamine, and neoarsphenamine may be permanently cured with single doses of 0.4 and 0.5 gm. per kilo of body weight of the amide of *N*-phenylglycine-*p*-arsonic acid.

Treatment of Relapses with Repeated Doses.

In the treatment of relapses with repeated doses, the chief factors of importance to be considered are first, the severity and type of the infection and the general physical condition of the rabbit. If the relapse is long standing with chronic indurated lesions, a single dose of the drug may not penetrate the lesions sufficiently or operate over the necessary length of time required for resolution of such lesions. Moreover, as is frequently the case with this type of chronic relapse, the physical state of the rabbit may be very poor and a single large dose

of the drug might under these conditions prove somewhat dangerous. Hence, from a consideration of these factors, it would seem that a system of small repeated doses, properly spaced, is the most rational system of treating chronic trypanosomal relapses of rabbits. The size of the initial dose or doses should be large enough to exert some influence upon the infection and its lesions, and subsequent doses should be given before the effect of the previous dose has completely worn off. Consequently a knowledge of the general effect and duration of action of various sized doses of this arsenical, as well as accurate and frequent clinical observations of the rabbits under treatment, is essential to a satisfactory outcome with this system of therapy. In this connection, it is important to bear in mind that rabbits as well as other animal species exhibit a high degree of tolerance for the drug as has been shown elsewhere,² and this attribute which exists in rabbits infected with trypanosomiasis as well as in non-infected animals may be used to great advantage in the treatment of trypanosomiasis, both of the initial infections and of the relapses. Our experience in handling relapses with repeated doses of this drug has been extremely varied because we have purposely endeavored to ascertain what might be accomplished with various sized doses given at various intervals of time under the many different conditions imposed by the infection rather than an attempt to effect a cure in every case. We have selected certain typical protocols which will illustrate some of the essential points in question.

Fig. 26 shows the appearance of an 8 day relapse in a rabbit which had been treated 34 days before with 0.02 gm. of arsphenamine (German salvarsan) per kilo of body weight. The signs of the relapse increased rapidly and at the time of retreatment with the amide of *N*-phenylglycine-*p*-arsonic acid, the rabbit was not in a particularly good general condition. The face was markedly swollen and indurated; both eyes were closed, the base of both ears was involved in the inflammatory process, both testicles were swollen and indurated, and the prepuce was extremely swollen and congested. Three doses of 0.15 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid were given intravenously on successive days. There were no evidences of drug intoxication whatever, the general condition improved immediately, and

² Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1919, xxx, 417.

the clearing up of the clinical signs progressed rapidly as is seen in Fig. 27, which shows the appearance 6 days after treatment had been begun. At this time there remained only a slight swelling and induration of the face and a slight degree of resistance of normal size testicles. These residual signs had completely disappeared on the following day. Unfortunately no opinion of the ultimate therapeutic success of the treatment was possible in this particular case as the animal succumbed to a bacterial infection 24 days after treatment.

Fig. 28 illustrates the appearance of a relapse of 15 days duration in a rabbit that had been treated 49 days previously with 0.02 gm. of neoarsphenamine (German neosalvarsan) per kilo of body weight. There was a very marked swelling of the face and the base of the ears, particularly of the left, an indurated swelling of the eyelids with crust formation, a purulent exudate, loss of hair and complete closure of both eyes, a mucoserous nasal discharge, and a moderate involvement of the genitalia. In addition, the animal was very thin and weak. A dose of 0.5 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid per kilo was given intravenously and was followed by a marked general improvement and gradual disappearance of the signs of the infection with an accompanying gain in weight, although the rabbit still remained weak and middle ear disease developed. 2 weeks after the first dose of the drug had been given, a second dose of 0.5 gm. per kilo was given intravenously with no evidences of drug intoxication but with a decided improvement in the animal's general condition, although the local condition in the right middle ear continued to progress, causing a marked twisting of the head to the right. In the course of recovery from the trypanosomal infection, a large portion of diseased skin over the bridge of the nose and about the eyes gradually desquamated, leaving a healthy looking but bare surface denuded of hair. The growth of new hair in these areas was quite remarkable as is shown in Fig. 29. There was no later evidence of trypanosomiasis observed in this rabbit either clinically or at autopsy, and despite the middle ear abscess which was eventually the cause of death 59 days after the treatment of the relapse, the general physical condition remained excellent.

We have selected two examples of the treatment of nagana relapses of a more prolonged and chronic type which are generally considered to be much more difficult of permanent cure than the types previously

considered in this section. The first of these rabbits was originally treated intravenously with 0.2 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid per kilo of body weight which sufficed to clear up all signs of the infection. However, 27 days later, the diagnosis of a probable relapse was made because of signs about the eyelids, and although at the end of a week's time, the clinical signs of trypanosomiasis were outspoken, the relapse was allowed to progress and the rabbit was not treated until 22 days after the first signs had been noted. The appearance of the head of the rabbit on the day of retreatment is shown in Fig. 30. There was a marked edematous swelling of the face involving the cheeks and eyelids and the eyes were almost closed; both upper lips were extremely swollen and congested with some loss of hair and scab formation; the lower portions of the ears were considerably indurated and very hot to the touch and at the base of the right ear was a necrotic area the size of a silver half dollar. The prepuce was edematous, congested, and somewhat swollen and both testicles although not enlarged were indurated. The general physical condition of the rabbit was fairly good. Two doses of 0.4 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid given a week apart caused a marked improvement in the rabbit's condition as shown in Figs. 31 and 32, but 3 days after the second dose, there was a swelling of the left cheek and both left eyelids, with a slight mucopurulent discharge from the eye. The succeeding four doses (0.6, 0.5, 0.5, and 0.75 gm. per kilo) given over a period of 28 days were eventually effective in clearing up the clinical signs (Figs. 33 to 35), but a relapse ultimately occurred during the summer. Since the total amount of the drug administered was in our experience sufficiently large to cure a relapse of this duration and type, it would seem that the failure was due to the incorrect spacing of the doses which were too far apart. It is probable that if the first three doses had been given on successive or even on alternate days, a permanent cure would have been obtained.

As a contrast to the system of treatment of a relapse pursued in the preceding experiment which failed to effect a permanent cure, the following instance is given of the successful treatment of a chronic and very advanced relapse, a type of infection generally considered extremely difficult of cure. A rabbit infected with *Tr. brucei*, which had been treated in the spring, relapsed during the summer, and at the time of retreatment, about 3 months later, presented an extreme

picture of a long standing chronic relapse (Figs. 36 and 37). A large portion of the face including the area around both eyes and the bridge of the nose was practically devoid of hair. The skin had lost its elasticity, was quite firm, resistant, and indurated, and there were numerous small scabs over the surface, especially around the right eye. The base of the right ear was also swollen and indurated and there was a small raw thickened area on the right hind foot covered with reddish yellow scabs. The prepuce was markedly swollen and quite firm; both testicles although not enlarged were very firm and indurated. The rabbit was weak but in a fairly good state of nutrition. Treatment was begun with the injection of 0.75 gm. of the drug per kilo of body weight and repeated on the 4th and 6th days—a dose which we believed was unnecessarily large, yet the reaction and effect of which we wished to determine in this particular type of relapse.

The therapeutic effect of the drug upon the clinical lesions was most marked as is shown in Figs. 38 to 40. There was no loss of weight, the rabbit's general condition was very noticeably improved, and, moreover, there was no indication of any drug intoxication at any time. 1 week after treatment had been begun, most of the scabs about the base of the right ear, around both eyes, and over the face had scaled off, leaving a smooth, soft, elastic skin denuded of hair but otherwise quite healthy looking, and the involved area on the right hind foot had a similar appearance. The prepuce was practically normal and the testicles were rapidly becoming so.

The further changes in this rabbit during the succeeding 10 days were quite remarkable as are shown in Figs. 41 and 42. The eyelids, nose, face, and base of the right ear were no longer swollen or indurated and the rapid growth of hair in the bare areas was quite conspicuous. The affected area on the right hind foot was somewhat pinkish with a few yellowish pink scabs but with a beginning growth of fine hair. The external genitalia appeared normal, although on palpation the scrotum on both sides was still somewhat diffusely thickened and the testicles were atrophic. On the 11th and 14th days, two additional doses of 0.75 gm. per kilo of the drug were given intravenously for the purpose of determining any degree of acquired tolerance or any signs or symptoms of drug intoxication that these

additional large doses might cause, although from the point of view of the infection, we were confident that they were unnecessary. Apparently, however, they had no untoward effect whatever and the rabbit continued to improve very noticeably. By the end of the 3rd week, there were no residual lesions indicative of the trypanosomiasis infection except one or two small patchy areas below the inner canthus of each eye and just above the tip of the nose where the hair was thinner than elsewhere (Fig. 43), but in a short time, these areas had resumed a normal appearance. This particular rabbit has been held in the laboratory 3 years and there has been no return of the infection.

The treatment of trypanosomal relapses in rabbits with this compound depends largely upon two important factors, the general physical state of the rabbit and the extent and severity of the infection, and these conditions can in our opinion be most adequately handled by a system of repeated doses. Since the factor of tolerance for the amide of *N*-phenylglycine-*p*-arsonic acid is exhibited to a considerable degree in rabbits, the size of succeeding doses may be greatly increased if necessary and the number of dose repetitions may also be large. However, it is of essential importance that the succeeding doses should be repeated well before the effect of the previous dose has worn off.

Administration of the Drug by Routes Other than the Intravenous.

The great majority of experiments with the amide of *N*-phenylglycine-*p*-arsonic acid in the treatment of rabbit trypanosomiasis, including those described above, were carried out by the intravenous administration of the drug, since this route offers uniform and constant conditions from the point of view of actual administration as well as development of drug action. Under such conditions, a comparison of the effect of different sized doses and a final appraisal of the action of the drug from the point of view of a permanent cure may be more accurately made. However, since it may be desirable to employ routes of administration other than the intravenous, from the standpoint of actual usage and ease of manipulation, a few rabbits infected with *Tr. brucei*, of which the following are examples, were treated subcutaneously, intramuscularly, and *per os*, in order to ascertain the general effect and duration of certain selected doses upon initial infections and relapses.

Two rabbits with a 14 day nagana infection and showing well marked clinical signs of the disease were treated with 0.1 gm. of the drug per kilo of body weight subcutaneously. The acute inflammation and edema about the head and external genitalia were materially reduced and only slight signs remained 7 days after treatment, but on the 8th day, they became increased in the first rabbit. Consequently both animals were treated on the 8th and 10th days with 0.1 gm. per kilo, again subcutaneously. The clinical signs regressed completely in the first animal during the following week and were absent for 3 weeks, at which time they recurred with considerable severity; in the case of the second rabbit, in which the infection was still in abeyance at the time of the second and third doses, the clinical signs were absent for 6 days after the second treatment; they then recurred and steadily increased in severity and extent. Two other rabbits in this group were treated subcutaneously with a dose of 0.2 gm. of the drug per kilo of body weight which was not repeated. One rabbit was permanently cured and the other relapsed on the 36th day. A fifth animal with a long standing infection of 48 days duration showing marked chronic lesions about the head and external genitalia with considerable loss of weight was treated with 0.5 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid per kilo subcutaneously. There were no signs of drug intoxication and no inflammation or induration about the site of injection; the regression of lesions proceeded somewhat more slowly than after intravenous administration of the drug, but at the end of 2 weeks they had subsided and the animal appeared normal except for a few bald patches about the head. No further signs of trypanosomiasis were observed for 41 days, at which time the outer margin of the right ear showed signs of early involvement and 2 days later a second dose of 0.25 gm. per kilo, one-half the original dose, was given subcutaneously for the purpose of ascertaining the effect upon the local lesion and upon the general course of the infection of a single dose much smaller than the original one. There was a very noticeable improvement of the ear lesion on the following day. A portion of the scab scaled off and the induration of the entire margin was distinctly decreased; on the 5th day after treatment, the ear appeared normal. There were no further indications of a relapse but unfortunately the rabbit developed an acute, extensive ringworm and

had to be killed 35 days after the second dose so that the question of a permanent cure is problematical. The rapid healing of the local ear lesion, however, and the fact that the relapse was treated soon after its appearance, although with a comparatively small dose, and that as long a time as 35 days elapsed without signs of a relapse either clinically or at autopsy are all factors indicative of a probable cure.

Seven rabbits were treated by the intramuscular route of administration. Two animals which were treated 14 days after inoculation with *Tr. brucei* received single doses of 0.2 gm. of the drug per kilo. The speed of action of the drug upon the well marked characteristic clinical lesions of the infection as seen in these two animals was apparently quite comparable with that observed after intravenous administration, and a permanent cure was effected in both rabbits. Two other rabbits of the same series were treated with an initial dose of 0.1 gm. per kilo which was repeated on the 8th and 10th days. One animal was cured but the other showed marked lesions of the disease 6 days after the last dose and was treated again with three doses of 0.1 gm. per kilo of body weight given intramuscularly on successive days with a marked, though temporary effect. In this instance, it is probable that the infection was originally not cleared by the first three doses and consequently either the second course of treatment was not given soon enough to effect a permanent cure or the amount of drug was not large enough. Another rabbit was treated 42 days after infection with *Tr. brucei* with three doses of 0.1 gm. per kilo of body weight intramuscularly on successive days. At the time of treatment, the lesions about the ears, eyes, face, nose, and lips were extremely marked and the animal itself was in only a fair physical condition. Regression and healing of the lesions about the head took place very satisfactorily and there were no signs of drug intoxication, but a subcutaneous abscess developed at the site of injection in the lumbar region and gradually increased in extent. The rabbit was killed 25 days after treatment. There were no clinical signs of a relapse at this time and the autopsy findings were negative. Finally, two rabbits with severe relapses after previous treatment—one with 0.015 gm. per kilo of neoarsphenamine given intravenously and the other with three doses of 0.1 gm. per kilo of the amide of *N*-phenylglycine-*p*-arsonic acid given subcutaneously—were treated intramuscularly with

larger doses of the latter drug. The first, which received 0.5 gm. per kilo of body weight, showed a rapid regression and healing of the lesions of the infection and was permanently cured; the other rabbit was given an initial dose of 0.3 gm. per kilo of body weight and 3 days later 0.5 gm. per kilo with no sign of drug intoxication and a very marked improvement in the signs of the infection. The ultimate result of the intramuscular administration of the drug cannot be determined in this case, for the succeeding treatment 11 days later was by mistake given intravenously.

Finally, an example of the therapeutic administration of the amide of *N*-phenylglycine-*p*-arsonic acid by mouth may be given for completeness, although the number of trypanosomiasis rabbits treated by this route is too small to admit of any conclusion regarding the effect and final result of various sized doses. A severe initial infection of 8 days duration with well marked clinical signs was treated with a single dose of 0.75 gm. per kilo of body weight given *per os* immediately after a small dose of sodium bicarbonate. There were no untoward effects following the administration of the drug; the clinical signs of the infection regressed and healed in the usual time and the rabbit was permanently cured.

While the number of rabbits treated with the amide of *N*-phenylglycine-*p*-arsonic acid administered by the subcutaneous, intramuscular, and *per os* routes is too few to admit of final conclusions as to their comparative value and usefulness, they demonstrate that even relatively small doses of the drug are highly active therapeutically when given intramuscularly and to a somewhat less extent subcutaneously, while in one instance a large dose given by mouth cured a severely infected animal.

SUMMARY.

In the treatment of experimental trypanosomiasis of rabbits with subsequent appraisal of the value of the therapeutic agent used, there are certain experimental factors including uniform infecting strains of trypanosomes and the observation of general procedures of method and time of inoculation conditioned by the infection itself which must be taken into account. The conspicuous and characteristic clinical

signs and symptoms seen in rabbit trypanosomiasis serve as criteria of the severity and duration of the disease, and it is obvious that the infection should be well established before treatment is instituted. For the same reason, before the question of a permanent cure can be established, treated rabbits should be kept under observation for a sufficient period of time, which with the species of organisms that we have used is at least 3 months.

The therapeutic results with the amide of *N*-phenylglycine-*p*-arsonic acid were obtained in rabbits which showed well marked clinical signs of a definitely established disease, and in many instances the infection was extremely advanced and of prolonged duration. The five species which we have employed, *Tr. brucei*, *Tr. gambiense*, *Tr. equinum*, *Tr. equiperdum*, and *Tr. evansi*, are uniformly fatal in rabbits. With the usual acute, actively progressing infection of from 1 to 2 weeks duration produced by our strain of *Tr. brucei*, the drug has a curative range of from 0.2 to 0.35 gm. per kilo of body weight, when administered intravenously in single doses, or from one-third to one-half the minimal lethal dose. Of the twenty-nine rabbits treated with doses falling within this range, twenty-five, or 86 per cent, were permanently cured and there were no relapses observed with doses above 0.3 gm. The infection produced by our strain of *Tr. gambiense* is controlled by a slightly lower dose, since there were no relapses with single doses of 0.3 gm. and a single dose of 0.15 gm. effected a cure in one of three rabbits so treated. The therapeutic experiments with *Tr. equinum*, *Tr. equiperdum*, and *Tr. evansi* are too few to admit of final conclusions, but apparently from the evidence at hand, much the same curative range is operative in *Tr. evansi* infections, while larger doses or a different system of treatment should have been employed in the treatment of rabbits infected with our strains of *Tr. equinum* and *Tr. equiperdum*.

In addition to the ultimate curative results obtained with single doses within the curative range, it is important to consider the marked therapeutic action with smaller single doses, as shown by the rapid regression and healing of the clinical lesions of the acute infections produced by all five species of trypanosomes together with a marked improvement in the general physical state of the animal. Moreover, large single doses, above those of the so called curative range, caused no disturbance of a toxic nature and were apparently well borne.

A system of repeated dose therapy may be employed with advantage in the treatment of both initial and relapsed infections in rabbits, especially in those instances in which there is induration or even necrosis of tissues with weakness and emaciation of the animal host. The factor of time of repetition or the spacing of doses is in our experience as important as that of size of the dose employed and depends upon the rate, degree, and duration of action of the particular dose of the drug in question. Since the amide of *N*-phenylglycine-*p*-arsonic acid apparently possesses the power of tissue penetration to a marked degree, it is desirable to give the second dose within a short time after the first in order that it may have a full opportunity for the immediate and complete development of its action. The repetition of small doses such as 0.15 gm. per kilo of body weight on successive or alternate days has given successful results as regards both the immediate regression and healing of lesions and ultimate permanent cures in severe, chronic infections. It is possible, however, to administer increasingly large doses, if this is necessary, since infected as well as normal rabbits exhibit a remarkable tolerance to repeated large doses of the drug. The therapeutic activity of small doses administered intramuscularly is quite comparable with that observed after similar doses given intravenously, as indicated by the rate of regression and healing of clinical lesions, while such effects proceed somewhat more slowly after subcutaneous injections. Permanent cures have been obtained in *Tr. brucei* infection with intramuscular and subcutaneous administration of single doses of from 0.2 to 0.5 gm. of the drug per kilo of body weight and in other instances with three repeated doses of 0.1 gm. per kilo given intramuscularly. One severely infected rabbit which received 0.75 gm. per kilo *per os* immediately following a small dose of sodium bicarbonate was also cured.

The therapeutic experiments here reported represent only a portion of those carried out with *N*-phenylglycineamide-*p*-arsonic acid and the scope of the present paper does not permit a detailed description of the many phases of the experiments or a full discussion of the various factors involved and the results obtained, all of which we hope to publish at some future time.

EXPLANATION OF PLATES.

The figures are reproductions of untouched photographs illustrating the effects produced upon experimental trypanosomiasis of rabbits by treatment with *N*-phenylglycineamide-*p*-arsonic acid. Objects are represented at about two-thirds their natural size, except where otherwise specified.

Figs. 1 to 17 illustrate the effect of single dose treatment upon acute and subacute initial infections.

PLATE 17.

FIG. 1. *Tr. brucei*. Acute infection of 15 days duration. Day of treatment with 0.35 gm. per kilo of body weight given intravenously. There is marked swelling of the face, eyelids, and base of both ears. General condition of animal good.

FIG. 2. 1 week after treatment. Appearance of rabbit normal. Permanently cured.

PLATE 18.

FIG. 3. *Tr. brucei*. Acute infection of 15 days duration. Objects represented at their natural size in Figs. 3 and 4. Day of treatment with 0.2 gm. per kilo of body weight given intravenously. The vulva and anus are markedly swollen and congested and are somewhat indurated. General condition of rabbit is good.

FIG. 4. 5 days after treatment. Rabbit permanently cured.

FIG. 5. *Tr. brucei*. Very severe, acute infection of 23 days duration. Day of treatment with 0.6 gm. per kilo of body weight given intravenously. The face, eyelids, and the lower portions of the ears are extremely swollen and indurated. The general physical condition of the rabbit is fairly good.

PLATE 19.

FIG. 6. 1 week after treatment. Slight residual fullness of the face. The hair over the nose and both upper lids is coming off. Animal's general condition is good.

FIG. 7. 29 days after treatment. Permanently cured.

PLATE 20.

FIG. 8. *Tr. gambiense*. Severe subacute infection of 36 days duration. Objects represented at their natural size in Figs. 8 to 11. Day of treatment with 0.2 gm. per kilo given intravenously. Both testicles are enlarged to about three times normal size and the scrotum is thickened, indurated, and cyanotic; on the right there are three large superficial ulcerations with scab formation. The testicles and cords are also enlarged and indurated. Rabbit's general condition is fairly good.

FIG. 9. 5 days after treatment. Scrotal scabs are separating.

FIG. 10. 19 days after treatment. There is a small residual adherent scab at the tip of the right scrotum. Testicle negative.

FIG. 11. 111 days after treatment—the day rabbit was discarded as permanently cured. External genitalia negative.

PLATE 21.

FIG. 12. *Tr. equiperdum*. Severe subacute infection of 36 days duration. Day of treatment with 0.3 gm. per kilo of body weight given intravenously. Rabbit is very thin and weak. There is marked swelling of the face with moderate involvement of the ears. Both eyes, especially the right, are partially closed and the upper eyelids are swollen and reddened.

PLATE 22.

FIG. 13. 5 days after treatment. There are no signs of trypanosomiasis and the animal's general condition is excellent. Ultimate recurrence.

PLATE 23.

FIG. 14. *Tr. evansi*. Severe subacute infection of 36 days duration. Day of treatment with 0.3 gm. per kilo of body weight given intravenously. Both ears are involved nearly to the tip, the left more than the right; the face is swollen with thickening of the tissues over the bridge of the nose and of the lips. All four eyelids are swollen and reddened; there is a mucopurulent discharge from both eyes, which are almost closed, and numerous yellowish scabs adherent to the lids. General physical condition of rabbit is good.

FIG. 15. 5 days after treatment. Very marked improvement. General condition good.

PLATE 24.

FIG. 16. 15 days after treatment. Scabs over end of nose and lips have desquamated. Beginning growth of hair.

FIG. 17. 19 days after treatment. The hair over the nose and lips is growing rapidly. Rabbit's condition is good with a considerable gain in weight. Permanently cured.

PLATE 25.

Figs. 18 to 23 illustrate the effect of repeated dose therapy upon a chronic initial infection.

FIG. 18. *Tr. brucei*. Extremely severe chronic infection of 48 days duration. Day of beginning treatment with three doses of 0.2 gm. per kilo of body weight repeated at 24 hour intervals and given intravenously. There is a marked and extensive indurated swelling of the tissues of the ears, face, eyelids, nose, and

lips with a purulent exudate from the eyes and nose. Rabbit is weak and emaciated.

FIG. 19. 6 days after treatment had been begun. Marked improvement in the local clinical signs of the infection and in the rabbit's general condition.

PLATE 26.

FIG. 20. 6 days after treatment had been begun.

FIG. 21. 13 days after treatment had been begun.

PLATE 27.

FIG. 22. 21 days after treatment had been begun. There is a very marked growth of hair in the bald areas of the face and ears.

FIG. 23. 40 days after treatment. Permanently cured.

PLATE 28.

Figs. 24 and 25 illustrate the effect of single dose treatment upon an acute relapsed infection.

FIG. 24. *Tr. brucei*. Acute relapse of 7 days duration. Day of treatment with 0.5 gm. of the drug per kilo of body weight given intravenously. The entire left side of the face including the eyelids and ears is swollen. Good general physical condition.

FIG. 25. 1 week after treatment. Rabbit appears entirely normal. Permanently cured.

PLATE 29.

Figs. 26 to 44 illustrate the effect of repeated dose treatment upon acute, subacute, and chronic relapsed infections.

FIG. 26. *Tr. brucei*. Severe acute relapse of 8 days duration. Day of beginning treatment consisting of three doses of 0.15 gm. per kilo of body weight repeated at 24 hour intervals and given intravenously. The face is markedly swollen and indurated and both eyes are closed. The base of both ears is similarly involved. General condition of the rabbit is fair.

FIG. 27. 6 days after treatment had been begun. Slight residual swelling over the bridge of the nose. General condition excellent. Probable permanent cure.

PLATE 30.

FIG. 28. *Tr. brucei*. Severe relapse of 15 days duration on the day of beginning treatment with 0.5 gm. per kilo of body weight given intravenously and repeated 14 days later. There is marked involvement of the face, ears, eyelids, nose, and lips and a mucoserous nasal discharge. Animal very thin and weak.

FIG. 29. 49 days after treatment. Appearance of rabbit normal except for the twisting of the head to the right. Probable permanent cure.

PLATE 31.

FIG. 30. *Tr. brucei*. Severe relapse of 22 days duration on the day of beginning treatment with 0.4 gm. per kilo of body weight given intravenously. The face, base of both ears, eyelids, nose, and upper lips are markedly swollen and edematous and there are thick scabs on the lips.

FIG. 31. 7 days after treatment had been begun. There is a marked improvement in the clinical signs.

PLATE 32.

FIG. 32. 9 days after treatment had been begun. Improvement continues with increase of weight.

FIG. 33. 15 days after treatment had been begun. Clinical signs of the infection have recurred.

PLATE 33.

FIG. 34. 21 days after treatment had been begun. Marked improvement with desquamation of the scabs and necrotic tissue in the involved area. General condition excellent.

FIG. 35. 28 days after treatment had been begun. Further improvement with rapid return to normal appearance. Ultimate relapse.

PLATE 34.

FIG. 36. *Tr. brucei*. Advanced chronic relapse of about 3 months duration on the day of beginning treatment with repeated doses of 0.75 gm. per kilo of body weight given intravenously. There is marked involvement of the face, eyelids, base of ears, and nose with loss of hair and scab formation. Rabbit is weak but not markedly emaciated.

FIG. 37. *Tr. brucei*. Day of treatment. Objects represented at their natural size. The prepuce is markedly swollen and indurated; the testicles and scrotum are very firm and indurated although not enlarged.

PLATE 35.

FIG. 38. 3 days later.

FIGS. 39 and 40. 1 week later. The appearance of the head and the external genitalia is normal except for the absence of hair and a few tiny scabs around the right eye. In Fig. 40 objects are represented at their natural size.

PLATE 36.

FIG. 41. 12 days later. There is a growth of fine hair on the bald patches about the head.

FIG. 42. 17 days later. Rapid growth of hair continues.

PLATE 37.

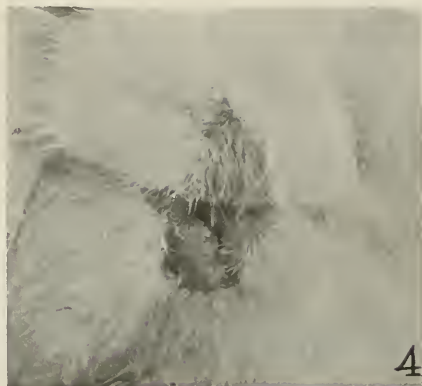
FIG. 43. 22 days later. Rabbit's appearance is entirely normal except for a tiny bald spot below the inner canthus of the right eye.

FIG. 44. 1 year later. Animal permanently cured.

682



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



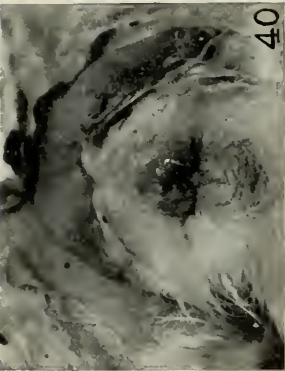
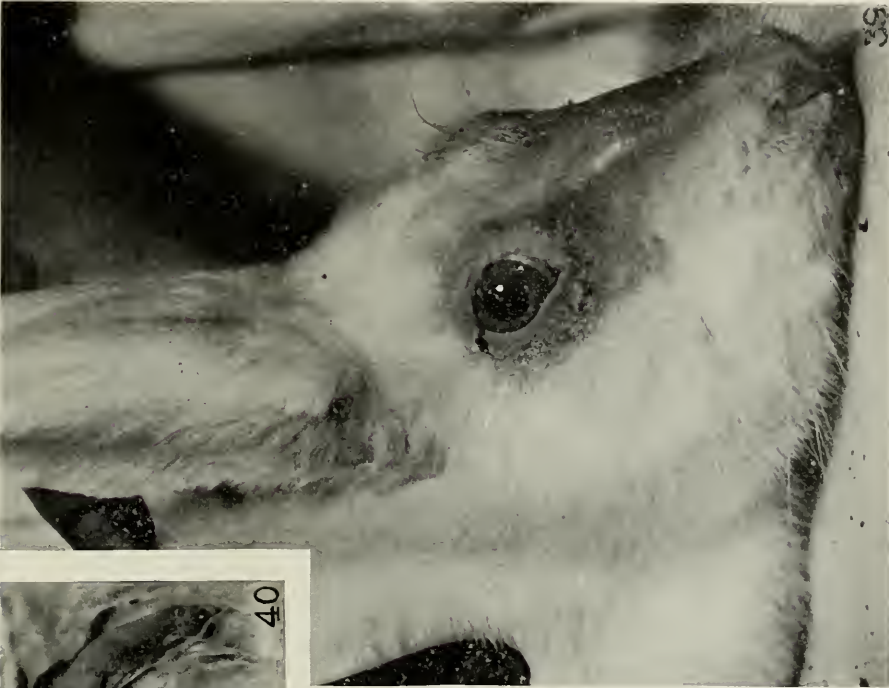
(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)

CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

BIOLOGICAL SERIES. IV.

THE ACTION OF N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID UPON SPIROCHETE INFECTIONS.

BY WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 38 to 44.

(Received for publication, June 18, 1919.)

A second group of conditions upon which the action of *N*-phenylglycineamide-*p*-arsonic acid has been studied is the infections produced in laboratory animals by spirochetes of the *recurrens* group and by *Treponema pallidum*. Early in the course of these studies, it was found that the action of the drug upon these organisms was not so pronounced as in the case of the trypanosomes. Accordingly, the experiments which were carried out were designed more for the purpose of studying the range and character of the action than as attempts to cure the infections.

Blood Spirochetes.

The action of A 63 upon the spirochetes of relapsing fever was studied in both rats and mice infected with *Sp. obermeieri* and with *Sp. novyi*. The animals for these experiments were inoculated intraperitoneally with blood suspensions of the organisms given in such doses as would produce an infection of the desired character—usually an infection which would show a few spirochetes in the peripheral blood within 24 hours after inoculation. The animals were then treated by intraperitoneal injection of single doses of the drug and the results followed from day to day by examination of tail blood under dark-field illumination. Finally, at the end of 60 days, surviving

animals were reinoculated as a means of determining the presence or absence of immunity such as usually develops in the course of untreated infections which terminate in spontaneous recovery, or in treated animals which have not been cured, and is absent in those which have been cured.

The results obtained from the treatment of rats infected with either of the spirochetes mentioned were almost entirely negative. The blood of these animals could not be cleared of spirochetes except by the use of doses of the drug which were nearly always fatal (1.5 gm. per kilo), while smaller doses exercised very little influence upon the course of the infection.

The action of A 63 appeared to better advantage in mice, however, due perhaps to the greater tolerance of these animals for the drug. With an average infection, the dose required to clear the blood of spirochetes temporarily was 1.5 gm. per kilo, or approximately the same as that found to be necessary in the case of rats, but no lasting effect was produced until the dose of the drug used reached upwards of 2 gm. per kilo of mouse, or the largest dose which could be used with safety.

The general character of the therapeutic effects produced may be seen by reference to Table I which shows the results obtained from the treatment of one series of mice with doses of A 63 ranging from 1.5 to 2.25 gm. per kilo. The results of blood examinations are given for the 1st week only with the final result obtained from reinoculation at the end of 60 days.

The infection in these mice might seem to have been a rather severe one since all four controls died within 8 days but the results obtained with the treated mice were of an average character. It will be seen from an examination of this table that the course of the infection was modified in all cases. Usually the progress of the infection was checked rather abruptly and the peripheral blood freed of spirochetes within 24 hours, or by crisis as it were. In a few instances (Mice 6 and 12), this effect was accomplished more slowly; the progress of the infection was first arrested and the infection then terminated by lysis instead of by crisis. In still other animals, the effect of the drug was manifested only by a reduction in the severity of the infection as indicated by the numbers of organisms in the peripheral blood. These effects were not always lasting but were followed in some instances by one or more slight relapses.

When the surviving mice were reinoculated at the end of 60 days, an interesting condition was found in that some mice known to have shown a recurrence of spirochetes in the peripheral blood were as susceptible to reinoculation as those in which no relapse had been observed. While the untreated controls in this experiment were all

TABLE I.

Results Obtained from the Treatment of a 24 Hour Infection of Sp. obermeieri in Mice.

Dose per kilo.	No. of mouse.	Results of blood examinations on days following inoculation and treatment.								Blood on days after reinoculation on 60th day.	
		1	2	3	4	5	7	8	60	2	3
gm.											
2.25	1	+	-	-	-	-	-	-	-	+	++
	2	+	-	-	-	-	-	++	-	+	++
	3	+	-	-	-	-	-	-	D.		
	4	+	-	-	-	-	+	-	"		
	5	+	-	-	-	-	D.				
2.0	6	+	+	+	-	-	-	-	-	-	++
	7	+	-	-	-	-	-	-	-	-	++
	8	+	-	-	-	-	-	-	-	-	++
	9	+	-	-	-	-	-	-	-	-	+
	10	+	-	-	-	-	+	+	-	-	++
1.75	11	+	-	-	-	-	D.				
	12	+	+	+	-	-	+	-	D.		
	13	+	-	-	-	-	++	-	"		
	14	+	-	+	++	-	-	-	"		
	15	+	-	-	-	-	-	-	"		
1.5	16	+	-	-	-	-	++	-	-	-	-
	17	+	+	++	-	-	-	-	-	-	-
	18	+	-	++	-	-	++	++	-	-	-
Controls.	19	+	+++	++++	++++	-	D.				
	20	+	+++	++++	++++	-	"				
	21	+	+++	++++	++++	-	-	D.			
	22	+	+++	++++	++++	-	-	"			

In the tables - indicates no spirochetes found, + few spirochetes present, ++ spirochetes fairly numerous, +++ spirochetes numerous, ++++ spirochetes present in large numbers, D. dead.

dead, the negative results obtained with the mice given only 1.5 gm. of the drug per kilo served as checks against the positive results obtained with the other animals.

Relapse and spontaneous recovery without immunity are almost a paradox, but there are a number of suggestions which occur to one as to how or why such anomalous conditions might arise. Whatever the explanation offered, however, it is obvious that the drug has had its effect even in these relapses and that any immunity developed was either so slight or of such short duration that these mice reacted to reinoculation after 2 months, just as if they had been promptly and completely freed of the spirochetes by the administration of the drug.

In estimating the curative effects of A 63, therefore, one can speak with assurance only of the results of reinoculation. On this basis, it was found that mice infected with *Sp. obermeieri* could be cured with doses of A 63 ranging from 1.5 gm. per kilo of mouse upwards, but with no dose of the drug were we able to obtain cures in more than 75 per cent of the animals treated.

Treponema pallidum.

The action of drugs upon syphilitic infections has usually been studied by the use of some form of the infection which can be produced in rabbits by inoculation with *Treponema pallidum*, and these experimental infections have been used in various ways by different investigators. The lesions of the eye, testicle, and skin have all proved of some value but the scrotal chancre is generally regarded as the most serviceable lesion of the group. The effects produced by drugs upon these infections may be estimated conveniently in three ways: first, by the changes produced in the lesions themselves; second, by the effects upon the organisms present in the lesions; and finally, by the duration or permanence of any changes which may be induced.

In this way, the action of A 63 upon scrotal chancres was studied in a series of twenty-nine rabbits. The lesions were measured with calipers and in most instances were photographed before treatment and at various intervals thereafter in order to determine as accurately as possible the character, extent, and rate of the changes which took place. In like manner, the lesions were aspirated and the fluid was

examined by dark-field illumination before treatment and at intervals such as 1, 3, 5, 7 days, etc., after treatment as a means of following the effects upon the spirochetes. The treated animals were kept under constant observation for such periods of time as the purpose of the experiment demanded, which varied from 1 to 7 months with different animals. For the most part, treatment was carried out by the use of single doses of the drug given intravenously; one series of animals was treated by subcutaneous administration, a second by intramuscular, and a third by the use of repeated small doses of the drug given intravenously. Some study was also made of the action of the drug when given by mouth.

The nature of the results obtained from these experiments may be seen by reference to Table II which contains the abbreviated protocols of twelve rabbits treated by intravenous administration of single doses of A 63 ranging from 0.1 to 0.5 gm. per kilo of body weight.

In order to make these results more intelligible, certain details of the experiments must be supplied. Rabbit 1 of this series was inoculated November 20, 1916. The chancres developed rapidly and at the end of 50 days when treatment was carried out, they measured 2.4 by 2.4 by 1.5 cm. (8.64 cc.) and 2.6 by 2.3 by 1.6 cm. (9.568 cc.) on the right and left respectively. On the day following treatment, the chancres were somewhat edematous, and while a few non-motile spirochetes could still be found, they had disappeared entirely by the end of 48 hours and were never found again. The chancres regressed rapidly for 2 weeks after treatment, when the crusts were torn from the ulcers by the animal. The wounds became secondarily infected and healing was somewhat delayed. The animal was still negative when discarded 100 days after treatment.

The second rabbit of this group requires but little comment. It was an animal with moderately large chancres which reacted to treatment precisely as did Rabbit 1. This animal died from hemorrhagic septicemia 6 days after treatment.

The next two rabbits of the series present an important contrast in the effects produced by the drug. Rabbit 3 was inoculated November 20, 1916, and treated January 9, 1917. At the time of treatment the chancres measured 1.7 by 1.55 by 1.25 cm. (3.294 cc.) on the right and 2.1 by 1.8 by 1.5 cm. (5.67 cc.) on the left. They were markedly

TABLE II.

Results Obtained from the Treatment of Rabbits Infected with T. pallidum.

Dose per kilo.	No. of rabbit.	Testicle.	Chancres at time of treatment.		Results of treatment.						Length of observation.
			Volume.	No. of spirochetes.	Spirochetes.				Chancres.		
					24 hrs.	1 wk.	2 wks.	Recurrence.	Time of healing.	Recurrence.	
gm.			cc.						wks.		days
0.5	1	Right.	8.64	++	—	—	—	No.	5	No.	100
		Left.	9.568	++++	+	—	—				
	2	Right.	5.304	+++	—	D.					
		Left.	6.16	++	—						
0.4	3	Right.	3.294	+++	—	—	—	No.	3	No.	207
		Left.	5.67	++++	—	—	—				
	4	Right.	8.1	++++	—	—	—	Yes.	5	No.	147
		Left.	6.799	+++	+	—	—				
0.3	5	Right.	1.89	++++	—	—	—	No.	3	No.	207
		Left.	3.315	+++	—	—	—				
	6	Right.	3.956	+++	—	—	—	Yes.	3	No.	207
		Left.	2.925	++++	—	—	—				
0.2	7	Right.	1.3	+++	—	—	—	No.	3	No.	85
		Left.	3.22	++++	+	+	—				
	8	Right.	3.47	++++	+	+	—	Yes.	{ Not healed.	No.	85
		Left.	3.31	++++	+	—	—				
	9	Right.	4.817	++++	+	—	+++		D.		
		Left.	2.755	++++	+	++	+				
	10	Right.		++	++++	+	+	{ Never clear.	3	{ On left.	D.
		Left.	3.27	+++			++				
0.1	11	Right.	2.38	+++	++++	—	—	Yes.	2	Yes.	72
		Left.		No lesion.							
	12	Right.	1.586	+++	++	+	++	{ Never clear.	{ Not healed.	Yes.	
		Left.	1.42	+++	++		+				

indurated and growing very actively (Fig. 1). Spirochetes were numerous. On aspiration 24 hours later, there was a marked increase in the fluid content of the lesions and no spirochetes could be found nor were they observed at any subsequent examination. The lesions regressed rapidly and at the end of 3 weeks were completely healed with moderate residual thickening in the region of the scars on both sides (Figs. 1 to 4). This rabbit was kept under constant observation for 207 days during which time no lesions, local or general, were observed.

Rabbit 4 was treated 40 days after inoculation. On the day of treatment, the chancres measured 2.5 by 1.8 by 1.8 cm. (8.1 cc.) on the right and 2.1 by 1.85 by 1.75 cm. (6.799 cc.) on the left and were of essentially the same character as those of Rabbit 3 (Fig. 5). For the first 2 weeks, the effects of treatment appeared to be about the same as in Rabbit 3. Regression then proceeded more slowly and the lesions were not completely healed until the end of the 5th week (Figs. 5 to 8), and while there was considerable residual thickening about the scars, no spirochetes could be found. This diffuse thickening gradually diminished, but translucent, glistening, and slightly indurated patches developed at the site of the scars (Fig. 9), and on the 52nd day, numerous actively motile spirochetes were found in these patches. This condition persisted with but slight change for several weeks and then cleared up, as shown in Figs. 9 and 10. The point to be noted here is that although spirochetes were present in the scrotum of this animal at the site of the original lesions, the associated lesions were very slight and showed almost no tendency to growth.

Coming to a still smaller dose of the drug, we again have two animals in which the effects produced were somewhat different. Rabbit 5 was treated 50 days after inoculation. The chancres at this time measured 1.4 by 1.35 by 1 cm. (1.89 cc.) and 1.7 by 1.5 by 1.3 cm. (3.315 cc.) on the right and left respectively. They were both actively growing and well indurated chancres; the one on the right showed a small depressed ulcer, while that on the left was not ulcerated. Spirochetes were numerous on both sides. The results of treatment were essentially the same in all respects as those with Rabbit 3. During 207 days observation, neither spirochetes nor lesions of any kind could be found.

Rabbit 6 was inoculated on November 24, 1916, and treated 46

days later. The chancres in this animal measured 2.15 by 1.6 by 1.15 cm. (3.956 cc.) and 1.95 by 1.5 by 1 cm. (2.925 cc.). Spirochetes disappeared and healing of the chancres took place in the usual manner. There was no sign of relapse for 7 weeks after treatment when several small nodules not more than 1 mm. in diameter appeared in the region of the scar and along the vessels in the dorsal fold of the scrotum on the left. Puncture of these nodules showed numerous actively motile spirochetes. Similar lesions developed later on the right. This animal was kept under close observation for 23 weeks after these lesions appeared, during which time the lesions showed very little change, until they finally regressed and disappeared.

In the next group of rabbits, there were four animals which gave a variety of results. Rabbit 7 responded to treatment by a gradual disappearance of the spirochetes and by rapid regression and healing of the lesions which was almost complete in 3 weeks (Figs. 11 to 14), and remained negative during a period of 85 days observation.

Rabbit 8 also showed a reduction in the spirochetes, and for a short time near the end of the 2nd week after treatment, no spirochetes could be found. In the meantime the chancres had decreased to less than half their original size, but at this point regression ceased. The chancres did not grow again, however, but after remaining stationary for a week or so, underwent spontaneous regression.

Rabbits 9 and 10 behaved still differently. At no time were the lesions of these animals free from spirochetes with the exception of one chancre of No. 9. Nevertheless, the chancres all regressed at a normal rate, and in No. 10, they were healed at the end of 3 weeks with slightly thickened, glistening patches at the site of the scars (Figs. 15 to 18). This rabbit lived only 36 days after treatment, but during this time, there was no renewal of the growth of the lesions, although numerous actively motile spirochetes were present all the while. The other rabbit, No. 9, died of hemorrhagic septicemia before the chancres had healed.

The final group of two rabbits gave results of much the same character as those of the preceding group. Rabbit 11 was inoculated November 1, 1916, but there was very little reaction for 2 months. The chancre on the right then developed rapidly and when treatment was carried out 97 days after inoculation (or about 40 days after the

beginning of the specific reaction) the main chancre mass measured 1.85 by 1.14 by 1.13 cm. (2.38 cc.). On the left, there was only a tiny nodule. Spirochetes disappeared from the chancre in 1 week after treatment and the lesion was healed with moderate residual thickening at the end of 2 weeks (Figs. 19 to 21). The animal was observed for 72 days during which time a small nodule developed in the deeper tissues of the scrotum beneath the scar. A similar nodule also appeared in the left scrotum but neither of these nodules grew and had almost disappeared at the time the animal was discarded.

Rabbit 12 was inoculated December 18, 1916, and treated 49 days later. The chancre on the right measured 1.48 by 1.24 by 0.87 cm. (1.586 cc.) and that on the left 1.41 by 1.2 by 0.84 cm. (1.42 cc.). They were both well indurated and actively growing chancres (Fig. 22). The effect of treatment was to reduce the spirochetes to a moderate extent and to cause considerable regression of the lesions lasting over a period of about 12 days (Figs. 23 and 24). Both chancres then began to increase in size (Fig. 25), in which respect the result differed from that usually obtained from the use of larger doses of the drug.

To the effects which have been described, one other type of reaction should be added; namely, that of the refractory animal or the refractory infection, which was encountered twice in our series of twenty-nine rabbits. The best example which we have of the infection which did not yield to treatment is that shown in Figs. 26 to 29. This animal was inoculated November 8, 1916. For 2 months, the growth of the chancres was rather slow and irregular, but after that they developed very rapidly and measured 1.82 by 1.57 by 1.3 cm. (3.715 cc.) and 1.75 by 1.7 by 1.35 cm. (4.016 cc.) 87 days after inoculation. Both chancres were markedly indurated and contained unusually large numbers of spirochetes. The rabbit was given three intravenous injections of A 63, 48 hours apart, each dose representing 0.1 gm. per kilo. The effect of this treatment was comparatively slight. The spirochetes were very little affected, but the chancres diminished in size for about 9 days with a maximum decrease of 0.4 cm. in all dimensions. They then began to increase in size and 3 weeks after treatment (Fig. 28) measured 1.67 by 1.93 by 0.9 cm. (2.901 cc.) and 1.5 by 1.37 by 0.9 cm. (1.85 cc.). The rabbit was then treated for the second time with a single dose of 0.3 gm. given intramuscularly

with an effect which was but slightly greater than that of the first treatment, the extent of which is shown in Fig. 29.

While the effects which have been described relate almost entirely to intravenous therapy, much the same results were obtained when the drug was given either subcutaneously or intramuscularly. The chief difference noted was that slightly larger doses of the drug were required by either of these routes to produce effects comparable with those obtained by intravenous administration. The drug was found to be active also when given by mouth, but this method of treatment was not used to a sufficient extent to warrant any statement as to its relative value.

Finally, one series of rabbits was treated by the use of repeated small doses of A 63 given intravenously, mainly for the purpose of getting an idea of the size of the dose and the interval between doses which would be required to maintain a continuous therapeutic effect. Without going into the details of these experiments, it may be said that the smallest dose which appeared to exercise a definite influence upon the infection was 0.1 gm. per kilo and that such doses could not be spaced more than 48 hours apart if the effect was to be made continuous. The experiments were not carried far enough to determine just how many such doses would be required to produce a given end-result.

From these experiments one may gain a fair impression of the effects produced by the amide of *N*-phenylglycine-*p*-arsonic acid upon experimental infections of *Treponema pallidum*. As seen by examination of fluid drawn from the lesions, the effect upon the spirochetes is to produce an impairment of motility or a complete loss of motion followed by degeneration and gradual disintegration so that the spirochetes eventually disappear from the lesions if the dose used is sufficiently large. One may be reasonably certain, therefore, that the drug possesses some measure of spirocheticidal action, but the dose required to produce such an effect as that described is comparatively large. At least 0.1 gm. per kilo is necessary to produce any appreciable effect upon the spirochetes; and the effect is usually not very pronounced until the dose used reaches upwards of 0.3 gm. per kilo, and even 0.4 gm. does not always insure a permanent disappearance of the infecting organisms.

The effect of the drug upon the lesions themselves is much more definite and apparently out of proportion to the effect produced upon the spirochetes. As we have seen, complete resolution and healing of scrotal chancres may be accomplished with doses as small as 0.1 gm. per kilo, and this is the usual result obtained with doses as large as 0.2 to 0.3 gm. whether spirochetes are destroyed or not. Further than this, the effects, as far as the lesions are concerned, appear to be more enduring. In our experience, lesions which have once healed, have shown little or no tendency to recur even though actively motile spirochetes were present. In a way, this peculiar type of action reminds one of the process of spontaneous control of *pallida* infections in rabbits; but it is not the same, and whatever the explanation of these effects may be, they are definitely referable to the action of the drug.

CONCLUSIONS.

To summarize the results obtained from these experiments, one may say that *N*-phenylglycineamide-*p*-arsonic acid is capable of exercising a very definite effect upon the course of infections produced by spirochetes of the *recurrens* group and by *Treponema pallidum*. It is more difficult to say, however, just how these effects should be interpreted. In the case of the blood spirochetes, the infection is ameliorated, and even though the spirochetes are not immediately destroyed, the infection is frequently brought to a termination which leaves the animal in a condition not unlike that produced by more powerful spirocheticidal agents. That is, the infecting organisms are either affected in such a way that they eventually die off or are destroyed by the host in such a way that no lasting immunity is developed in consequence of their destruction.

Apparently much the same type of reaction occurs in the treatment of rabbits infected with *Treponema pallidum*. It is possible that when very large doses of the drug are used, these organisms may be completely destroyed, but it is certain that in other cases, where complete healing of the lesions is accomplished as a result of treatment, the organisms are not destroyed. Moreover, it appears that such a result can be accomplished in the presence of numerous actively motile spirochetes, and once the effect of the drug has reached this

point, either the capacity of the spirochetes for stimulating reaction on the part of the tissues is lowered or else the reactivity of the tissues is reduced. At any rate, living spirochetes may remain in the tissues for considerable periods of time without giving rise to the usual tissue reaction which characterizes these infections.

With either group of organisms, therefore, *N*-phenylglycineamide-*p*-arsonic acid appears to act in a manner somewhat different from that of the usual spirocheticidal agents. While it does possess a considerable degree of spirocheticidal action, its chief effect is seen in the peculiar manner in which it modifies or controls the course of these infections.

Further than this, we shall not attempt to go at present. As set forth in the literature, the facts and principles upon which such experiments depend are so few as to offer almost no basis for an interpretation of these experiments. When we have had an opportunity of reporting our own experience in dealing with these infections, we may return to a consideration of the facts here reported.

EXPLANATION OF PLATES.

The figures are reproductions of untouched photographs illustrating the effects produced upon scrotal chancres of rabbits by treatment with *N*-phenylglycineamide-*p*-arsonic acid. Objects are represented at their natural size.

PLATE 38.

FIGS. 1 to 4. Rabbit 3. Effect produced by 0.4 gm. per kilo of body weight.

FIG. 1. Chancres at time of treatment.

FIG. 2. 1 week after treatment.

FIG. 3. 2 weeks after treatment.

FIG. 4. 3 weeks after treatment. The lesions are healed with some residual thickening especially noticeable on the right, but no spirochetes could be found.

PLATE 39.

FIGS. 5 to 10. Rabbit 4. A peculiar therapeutic effect following the administration of a dose of 0.4 gm. per kilo.

FIG. 5. Chancres at the time of treatment.

FIG. 6. Chancres regressing rapidly.

FIG. 7. 4 weeks after treatment. Chancres unhealed but still regressing slowly.

FIG. 8. 5 weeks after treatment. Chancres are healed but the scars are thickened and translucent, especially that on the right.

FIG. 9. 9 weeks after treatment. The lesions are still healed but scars are now represented by translucent, highly refractile plaques which contain numerous actively motile spirochetes.

FIG. 10. 147 days after treatment. The foci of infection have cleared spontaneously and the scrotum is normal.

PLATE 40.

FIGS. 11 to 14. Rabbit 7. The effect of a single dose of 0.2 gm. per kilo.

FIG. 11. Chancres at the time of treatment. That on the right is an un ulcerated globular mass.

FIG. 12. 1 week after treatment; very rapid regression of the lesions.

FIG. 13. 3 weeks after treatment. The chancre on the right has entirely disappeared; that on the left still shows a small thickened mass.

FIG. 14. 4 weeks after treatment. Resolution complete.

PLATE 41.

FIGS. 15 to 18. Rabbit 10. An instance of the healing of chancres with a single dose of 0.2 gm. per kilo while actively motile spirochetes were constantly present.

FIG. 15. Chancres at the time of treatment.

FIG. 16. 1 week after treatment; marked regression of the lesions.

FIG. 17. 2 weeks after treatment.

FIG. 18. 3 weeks after treatment. The lesions have practically disappeared, but actively motile spirochetes are fairly numerous in the scars of both sides. There was a slight recurrent patch in the left scrotum.

PLATE 42.

FIGS. 19 to 21. Rabbit 11. An effect which may be accomplished by a dose of 0.1 gm. in lesions which are easily influenced.

FIG. 19. Chancre at the time of treatment.

FIG. 20. 1 week after treatment.

FIG. 21. 2 weeks after treatment.

PLATE 43.

FIGS. 22 to 25. Rabbit 12. The extent of the effect usually produced by a dose of 0.1 gm. per kilo.

FIG. 22. Chancres at the time of treatment.

FIG. 23. 8 days after treatment.

FIG. 24. 2 weeks after treatment. The chancre on the left is healed, but there is a considerable mass of unresolved tissue in the scrotum; that on the right is increasing.

FIG. 25. 3 weeks later. Both chancres growing actively.

PLATE 44.

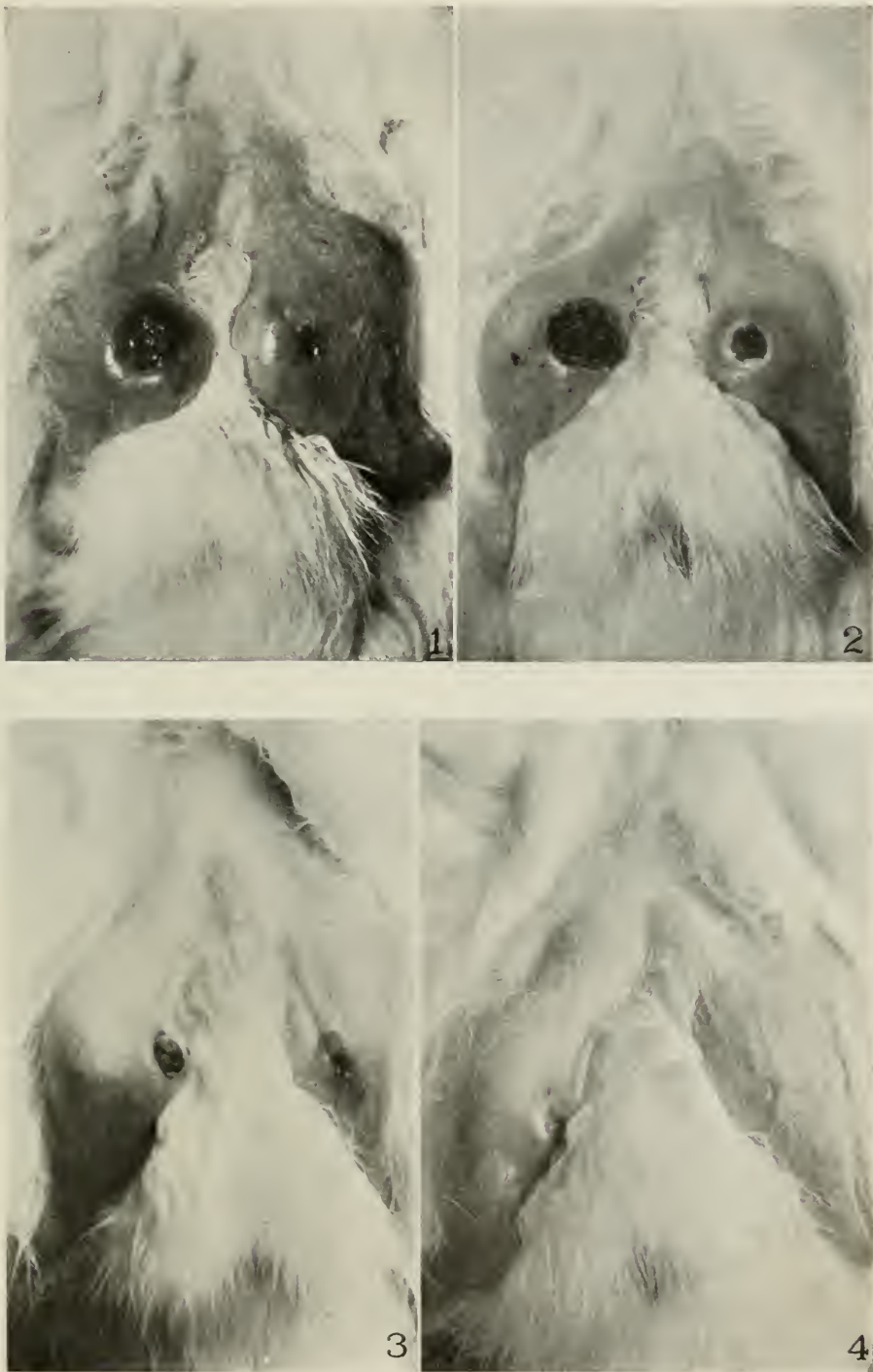
FIGS. 26 to 29. Chancres which proved refractory to treatment with this drug.

FIG. 26. Chancres at the time of first treatment which consisted of three doses of 0.1 gm. per kilo given intravenously at intervals of 48 hours.

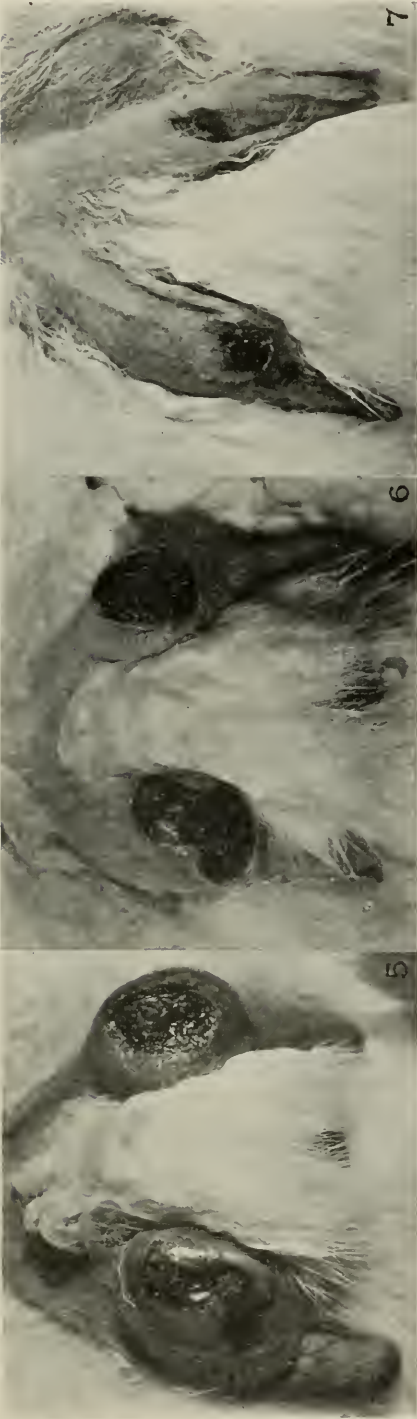
FIG. 27. 1 week after treatment had been commenced; practically the full extent of the reduction of the lesions is shown.

FIG. 28. 2 weeks later; the growth of the chancres in the interim is shown. Animal retreated with 0.3 gm. per kilo given intramuscularly.

FIG. 29. 1 week later; a decided effect is shown, but renewed activity set in almost immediately.



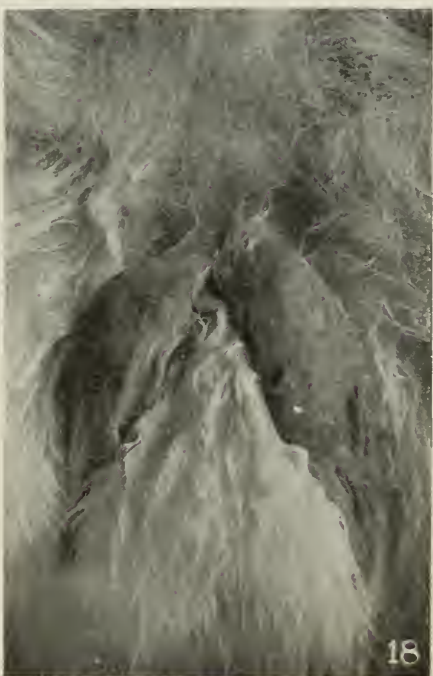
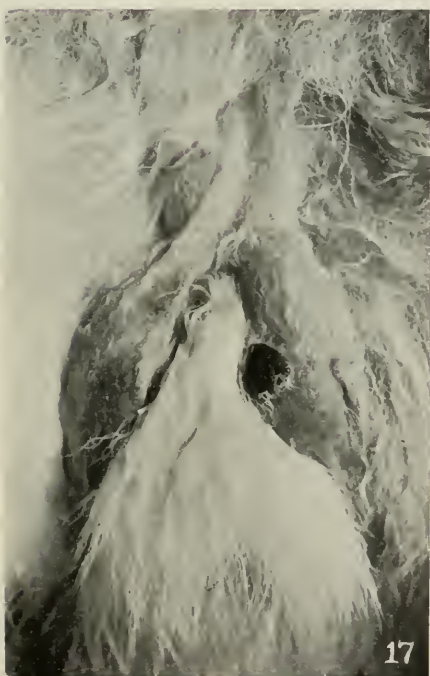
(Brown and Pearce: Trypanosome and spirochete infections.)



(Brown and Pearce: Trypanosome and spirochete infections.)



(Brown and Pearce: Trypanosome and spirochete infections.)



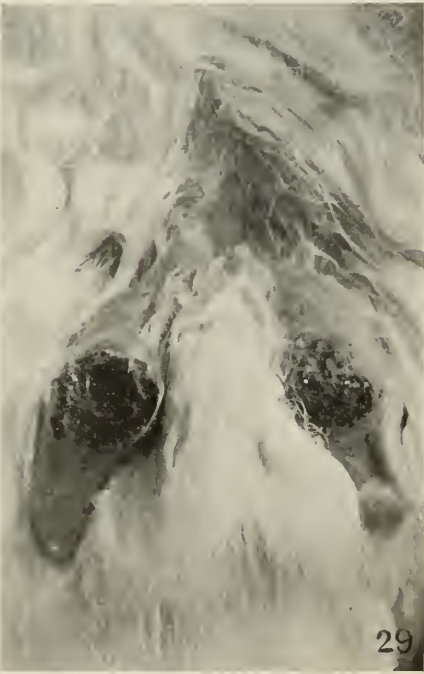
(Brown and Pearce: Trypanosome and spirochete infections.)



(Brown and Pearce: Trypanosome and spirochete infections.)



(Brown and Pearce: Trypanosome and spirochete infections.)



(Brown and Pearce: Trypanosome and spirochete infections.)

THE OCCURRENCE OF BACILLUS INFLUENZÆ IN THE NORMAL THROAT.

BY AGNES I. WINCHELL AND ERNEST G. STILLMAN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, July 15, 1919.)

The present study is a continuation of the work of Pritchett and Stillman on the occurrence of *Bacillus influenzae* in throats and saliva, with additional observations upon the media used and a study of the distribution of *Bacillus influenzae* among the personnel of two institutions.

Pritchett and Stillman¹ found that of 177 persons who gave no history of having had influenza, 74, or 42 per cent, harbored *B. influenzae* in their throats during November and December of 1918. The total incidence of influenza bacillus carriers among 231 normal and late convalescent individuals was 99, or 43 per cent. From 49 cases of uncomplicated influenza, *B. influenzae* was recovered in 41 instances, or 83 per cent, while from 43 cases complicated by bronchopneumonia this organism was cultivated in 40, or 93 per cent. Of 6 cases of bronchopneumonia, which were probably late cases of influenza, all showed influenza bacilli. The incidence of *B. influenzae* was much lower in 20 cases of lobar pneumonia, as only 11 cases, or 55 per cent, were positive. Pneumococci of Types III and IV, which are the groups usually encountered in normal mouths, were the types of pneumococci which were recovered from the cases of influenza complicated with bronchopneumonia.

Methods.

As in the previous study, the medium used in this investigation for the isolation of *Bacillus influenzae* was Avery's² oleate hemoglobin agar. 5 cc. of a 2 per cent solution of neutral sodium oleate were added to 95 cc. of meat infusion agar which made a final concentration of oleate of 1:1,000. 1 cc. of a suspension of rabbit red blood cells was then added while the agar was still hot. Plates were poured

¹ Pritchett, I. W., and Stillman, E. G., *J. Exp. Med.*, 1919, xxix, 259.

² Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxi, 2050.

containing about 15 cc. each. The medium was made fresh each day as the surface soon became dry.

The optimum hydrogen ion concentration for the isolation of influenza bacillus from the throat seems to be between pH 7.2 and 7.5. In every test of medium which was more alkaline than that having a pH of 7.5, unsatisfactory results were obtained. In one instance cultures were made on agar at pH 7.9 from 12 individuals who previously had been persistent carriers. Though the cultures were repeated, only 2 showed small colonies of *Bacillus influenzae*. But, when repeated a third time on medium with a pH of 7.5, 8 were found

TABLE I.

Comparison of Cultures upon Media of Different Hydrogen Ion Concentrations.

Culture No.	pH 8.0		pH 7.4	
	Series 1.	Series 2.	Series 1.	Series 2.
1	—	—	++	++
2	—	—	++	++
3	—	—	—	—
4	—	—	—	—
5	—	+	++	+
6	—	—	+	++
7	—	—	++	+
8	—	+	++	+
9	—	—	++	++
10	—	—	—	++
Total positive.	0	2	7	8

+ indicates colonies of *B. influenzae* present; ++ many colonies of *B. influenzae* present.

to be carrying influenza bacilli. Of 36 other individuals chosen at random, 14, or 38 per cent, were positive when agar at pH 7.4 was used, but only 4, or 11 per cent, with agar at pH 7.9. In Table I are given the results obtained in an examination of 10 individuals from whom both positive and negative plates had previously been obtained. Cultures were taken twice during the same day upon medium at pH 8.0 and at pH 7.4. No positive plates were found in Series 1 on medium with a pH of 8.0, but in Series 2, 2 plates contained several small colonies. On the other hand, Series 1 with 7.4 pH agar shows 7 positive plates, and Series 2, 8 positive plates.

Negative results are so consistently obtained with medium having a reaction above pH 7.5 that one is led to question the value of all negative results obtained upon medium with an unknown hydrogen ion concentration.

All cultures here reported, unless otherwise stated, were taken from the posterior wall of the pharynx. As shown by Table II cultures from the tonsils proved to be unsatisfactory. In a series of 77 individuals, 3 cultures were made from the throat—1 culture from each tonsil, and 1 from the posterior wall of the pharynx. In 4 individuals positive cultures were obtained on plates from all three sources. In 4 other individuals the cultures from the pharynx and from one or the other tonsil were positive. In 11 individuals only the culture from

TABLE II.
Comparison of Cultures from the Pharynx and the Tonsils.

Source of cultures.	No. positive.
Pharynx and both tonsils.....	4
“ “ one tonsil.....	4
Only tonsil.....	0
“ pharynx.....	11
Total No. of cases positive.....	19
“ “ “ “ negative.....	58
“ “ “ “ examined	77

the pharynx showed a growth of influenza bacilli. In no case were the organisms isolated from the tonsil and not from the pharynx. The influenza bacillus can unquestionably be isolated from the tonsils, but as there are so many other colonies on the plates made from the tonsils, the influenza bacilli are either overgrown or the colonies are so small that they are hard to detect. West tubes used in a few instances did not give any more satisfactory results than did the simpler method of pharyngeal cultures. Hence, it seemed unnecessary to subject the large group of individuals studied to the discomfort of nasopharyngeal cultures.

All organisms designated as *Bacillus influenzae* were demonstrated to possess the typical morphological and cultural characteristics of this organism; all cultures were proved to be definitely hemoglo-

binophilic. With the exception of staphylococci and diphtheroids, only Gram-negative organisms were encountered upon the oleate hemoglobin agar plates. In addition to the influenza bacillus, there appeared on these plates various types of Gram-negative cocci of the *Micrococcus catarrhalis* group and Friedländer's bacillus group, and also the unidentified bacillus, described by Pritchett and Stillman, which is differentiated from *Bacillus influenzae* by its hemolytic properties.

RESULTS.

Cultures were taken once a month for a period of 6 months from the throats of the personnel of the Laboratories and the Hospital of

TABLE III.
Incidence of B. influenzae in Convalescent and Normal Individuals.

	Nov. and Dec., 1918.			Jan., 1919.			Feb.			Mar.			Apr.			May.		
	Positive cases.			Positive cases.			Positive cases.			Positive cases.			Positive cases.			Positive cases.		
	No.	Per cent.		No.	Per cent.		No.	Per cent.		No.	Per cent.		No.	Per cent.		No.	Per cent.	
Normal individuals.....	177	74	42	127	17	13	122	42	34	109	56	51	96	33	34	86	34	39
Convalescents....	54	25	46	42	1	2	40	16	40	42	19	45	38	15	39	28	13	46
Total.....	231	99	43	169	18	11	162	58	36	151	75	50	134	48	36	114	47	41

The Rockefeller Institute in accordance with the method outlined above. It has been possible to follow but 84 of the original 231 individuals used in the earlier study. Of these, 15 have never yielded a positive result. 9 of 35 individuals positive upon the original examination have never since shown the presence of *Bacillus influenzae* in the throat. Of the total 84, 6 have been positive for 5 months and 13 for 4 months.

During the months from December, 1918, to June, 1919, as shown in Table III, the percentage of carriers in a group of approximately 150 individuals has been 43, 11, 36, 50, 36, and 41, respectively. In the previous study the individuals who gave a history of having had

influenza during the time of the epidemic were classed as convalescents. These same individuals have been followed during the complete period. 46 per cent were positive in November and December, 1918, 2 per cent in January, 1919, 40 per cent in February, 45 per cent in March, 39 per cent in April, and 46 per cent in May.

As previously mentioned, any culture medium which varies from the optimum hydrogen ion concentration is unsuitable for use in the isolation of *Bacillus influenzae* from the throat. The reaction of the medium used in November and December had a pH of 7.2, while that for March, April, and May had a pH of 7.4 and 7.5. The reaction of the medium employed during January and February was not tested, and it is apparent, therefore, that no importance can be attached to the low incidence during January.

TABLE IV.

Incidence of B. influenzae in Cases of Acute Respiratory Infections.

Date.	No.	Positive.	Per cent positive.
<i>1919</i>			
Feb.....	9	5	55
Mar.....	16	13	81
Apr.....	12	8	67
Total.....	37	26	70

As is seen from Table IV, *Bacillus influenzae* was recovered from 70 per cent of the persons suffering from acute respiratory diseases who were admitted to the Hospital of The Rockefeller Institute during the months of February to April. Thus it is seen that during the same months a larger percentage of positive cultures was obtained from patients suffering from acute respiratory diseases than from normal individuals.

During the latter part of January, an epidemic of influenza occurred in a girls' school. While the pandemic of influenza raged in the country surrounding this institution during the fall of 1918, a strict quarantine was maintained and only a few questionable cases developed. While the institution was still under strict quarantine, a teacher who had spent Sunday at home developed influenza. The

first cases developed in her classes, and within 10 days over half the students were ill. The epidemic then subsided as suddenly as it arose. On February 16 throat cultures were taken from 52 girls who were still in the infirmary. Of these, 20, or 38 per cent, showed *Bacillus influenzae*. In one cottage which had been under quarantine on account of trachoma since October, 1918, no cases of influenza developed. Of the 20 girls in this cottage, 5, or 25 per cent, showed *Bacillus influenzae*.

In a boys' orphan asylum where a lax quarantine had been maintained and which gave no history of an epidemic of influenza, 190 throat cultures were taken from boys whose ages varied from 10 to 12 years. *Bacillus influenzae* was recovered from 74, or 39 per cent.

DISCUSSION.

Whatever may be the etiologic relationship of *Bacillus influenzae* to epidemic influenza, there is little doubt of the significance of this organism as a secondary invader in this type of respiratory infection. Facts, therefore, concerning the distribution, occurrence, and persistence of *Bacillus influenzae* in the secretions of the respiratory tract are of importance in epidemiological investigation and in prophylaxis. In a study of the occurrence of *Bacillus influenzae* in throats and saliva during the epidemic of influenza last fall, Pritchett and Stillman found this organism present in 42 per cent of 177 healthy persons from whom no history of respiratory infection was obtainable. These observers found the same organism in the throats of convalescents from influenza in 46 per cent of individuals studied. In the same epidemic period Lord, Scott, and Nye,³ by cultural methods, demonstrated influenza bacilli to be present in the pharyngeal secretions of 76 per cent of 34 healthy men of the Harvard Student Army Training Corps. Opie and his collaborators,⁴ by cultural and mouse inoculation methods, found *Bacillus influenzae* in the mouths of 35.1 per cent of all healthy men examined at Camp Funston.

³ Lord, F. T., Scott, A. C., Jr., and Nye, R. N., *J. Am. Med. Assn.*, 1919, lxxii, 188.

⁴ Opie, E. L., Freeman, A. W., Blake, F. G., Small, J. C., and Rivers, T. M., *J. Am. Med. Assn.*, 1919, lxxii, 108.

These figures serve to indicate the wide distribution and prevalence of the organism during the severe epidemic of this acute respiratory disease.

Since the group of individuals studied by Pritchett and Stillman comprised for the most part the personnel of The Rockefeller Institute, it has been possible to make repeated cultural examinations of the throats of 84 of the same persons during the 6 months subsequent to the original observation. From this study certain facts of interest have been acquired concerning the duration of the carrier state, and the relative frequency of this organism in normal throats after the subsidence of the epidemic. The present study indicates that the percentage incidence of those harboring *Bacillus influenzae* in the upper respiratory tract is as great during the postepidemic period as it was during the influenzal epidemic. During December, 1918, to June, 1919, the percentage of carriers in a group of 150 individuals has averaged 41 per cent per month. This percentage incidence of healthy persons found to harbor influenza bacilli in their throats and saliva is approximately the same as that recorded by Pritchett and Stillman during the height of the epidemic. In addition, it is of interest that in a boys' orphan asylum in which no case of influenza had occurred during the epidemic, 39 per cent of throat cultures taken from 190 boys showed the presence of *Bacillus influenzae*. This percentage incidence of positive cultures is the same as that found in the examination of 52 convalescents from influenza in an institution for girls in which over half the personnel had suffered from the disease.

Furthermore, it is evident that *Bacillus influenzae* may persist in the throats of healthy carriers for a considerable period of time. In 6 instances individuals have had positive cultures on repeated examinations during 5 months, and in 13 other instances during a period of 4 months. In chronic non-tuberculous disease of the lungs *Bacillus influenzae* is known to persist for long periods of time in the secretions of the respiratory tract. The present study, however, demonstrates that in the absence of a focus of infection this bacillus may dwell on apparently normal mucous membranes for long intervals. Whether the organisms thus encountered represent a variety less parasitic than strains actually associated with pathologic lesions

is a problem requiring greater knowledge concerning the biologic differentiation of bacilli of the hemophilic group than is now available.

The technical difficulties incident to the isolation of *Bacillus influenzae*, particularly from the mixed flora of the upper respiratory tract, have been materially lessened by the use of oleate hemoglobin agar. The optimum hydrogen ion concentration for this medium is shown to lie between pH 7.2 and 7.5. Any variation beyond this range, particularly on the alkaline side, reduces markedly the suitability of this medium for growth of *Bacillus influenzae*. Oleate hemoglobin agar not only enhances the growth of the influenza bacillus, but by inhibiting the growth of other organisms, such as streptococci and pneumococci, greatly facilitates the isolation of *Bacillus influenzae* from a focus which harbors a wide variety of other bacteria.

CONCLUSIONS.

1. The frequency of occurrence of *Bacillus influenzae* in the throats of normal individuals has been as great in the period subsequent to the epidemic of influenza as it was during the period of the epidemic.
2. *Bacillus influenzae* may persist in the throats of healthy carriers for a considerable period of time.
3. A considerable proportion of individuals not exposed during the epidemic has been found to carry *Bacillus influenzae* in their throats.
4. Oleate hemoglobin agar has been found to be a very satisfactory selective medium for cultivation of *Bacillus influenzae* from the throat. To give optimum results it should have a reaction of from pH 7.2 to 7.5.

THE PATHOLOGICAL CHANGES IN THE TESTES IN EPIDEMIC PNEUMONIA.

BY RALPH G. MILLS, M. D.

(From the Department of Pathology of the Johns Hopkins University, Baltimore.)

PLATES 45 TO 50.

(Received for publication, July 12, 1919.)

Clinically orchitis or any other affection of the testes is uncommon in primary pneumonia or in pneumonia following either measles or influenza. Osler and McCrae (1) make no mention of this condition as following pneumonia. Equally rare is the atrophy of the testis following recovery from the disease, hence any extensive or permanent injury would be unlikely.

The only reference in literature to changes in the testis following pneumonia is an article by Cordes (2) in which he tabulates the findings in twenty cases of primary pneumonia, in patients between the ages of 21 and 60 years. No case was seen in which the spermatogonia were so badly damaged as to suggest that had the patient recovered there would have been no regeneration of these cells and restoration of function.

Sixty cases were studied. Pneumonia was the cause of death in every instance, but in some cases it was the sole factor, while in others it was preceded by measles or epidemic influenza. The changes in the body directly due to measles and influenza are little known and hence the extent of their influence in the present study must be determined by the indirect method of comparing the picture seen in primary pneumonia with that in pneumonia following measles and influenza. The presence of respiratory irritation and of lowered resistance to infection is well recognized and their importance in a given case becomes manifest with the advent of pneumonia or the awakening of some latent infection.

The bacteriology of the cases varied greatly. The presence and the importance of the hemolytic streptococcus in the first epidemic were generally recognized, but the part played by the Pfeiffer bacillus has been the subject of much discussion. Its presence was frequently noted in the measles epidemic, but no special attention was given it other than to consider it a secondary invader. It attracted attention during the influenza epidemic and the significance of its presence has been much studied.

During these epidemics lobar pneumonia caused by the pneumococcus has been largely replaced by other forms. A single organism was in some cases the apparent cause of the pneumonia, but in many instances two or more of the pathogenic bacteria were associated, the variety of combinations contributing greatly to the complexity of the problem. These cases may be classified into several groups on the basis of the etiological factors involved: (1) primary pneumonia—pneumonia occurring during the measles epidemic and due to the same organisms, but not preceded by an attack of that disease; (2) pneumonia following measles; (3) pneumonia following epidemic influenza. These have been further subdivided according to the occurrence of *Streptococcus hæmolyticus*, *Bacillus influenzae*, and the pneumococcus, both alone and in combination.

Complete tables of the findings in the cases, based on this grouping, have been made and are given below. The bacteriological findings in these cases are more complete than is usually found. Careful attention was given to the examinations and every effort was made to isolate and identify all organisms present.

With the development of the MacCallum stain (3), by which the Gram-negative organisms may also be identified in sections, the influenza bacillus has been added to several of these cases in which it was not found at previous examinations. In spite of all care it is more than likely that the findings do not report all the organisms present.

It was manifestly impossible to distinguish and study the effect of every organism present; hence the three supposed to be the most important were selected as the basis for comparison. In regard to the general infection and the etiology of the principal lesions, MacCallum (4, 5) has reached certain conclusions concerning the relative importance of the various organisms present in the different cases, and these have been followed in the present study.

The subjects were all soldiers in good health, recently passed through the rigid examination for admission to the Army.¹ The ages varied from 18 to 40 years. Presumably all individuals with any sort of chronic or debilitating disease had been excluded from the Army and the supposition is warranted that these men were in excellent health. This supposition is important in the light of the work of Weichselbaum and Kyrle (6) who have described changes in the testes in persons dying of chronic alcoholism, and of others who mention the effect of tuberculosis, syphilis, liver disease, and malignancy in causing somewhat similar lesions.

Syphilis is always an uncertain factor, but the age of the men, the absence of postmortem luetic signs, the nature of the Army entrance examination, and the continued medical supervision make this disease of less importance in this series than it would be in almost any other that could be secured.

Tuberculosis as an active process is almost negligible, those individuals with any but the most latent lesions having been previously excluded. Military training would have tended to render quiescent any latent infection or to lead to the subsequent elimination of the individual as unfit. In this series cavity formation was found in one case, but this was of such a nature that it had probably not been injurious for any considerable period of time. Presumably the usual number of healed tubercles was encountered that occurs in autopsies on persons dying of other diseases and hence are not considered to be of any importance in the present discussion. It is reasonably safe also to eliminate from this discussion any uncertainty referable to the possible insidious influence of diabetes, liver and kidney alterations, and all other chronic diseases.

The associated organisms were commonly staphylococcus and *B. mucosus capsulatus*. Neither of these is supposed to have been operative to an extent sufficient to contribute much to the pathological picture because of the probability that they were secondary invaders shortly before or possibly after death.

The length of time that the individuals had been in the hospital was variable. The anatomical diagnosis indicates the degree to which other related conditions prolonged or complicated the original picture

¹ The material studied was obtained by Professor W. G. MacCallum at Camps Sam Houston, Dodge, Dix, and Lee during the measles epidemic of 1917 and the influenza epidemic of 1918. The numbers of the autopsies to which reference is made agree with those used in his monograph published by The Rockefeller Institute for Medical Research (4) and in another to be published in the *Johns Hopkins Hospital Reports*, and are here repeated for convenience in making cross-reference.

of the pneumonia. Unfortunately the number of days in the hospital in some cases does not include the length of time covered by the primary disease. In a given instance this fact can be surmised by comparison with others of similar pathological picture. Roughly the severity of the injury corresponds directly with the duration of the pneumonia as closely as with any other single variable factor.

General Description of the Testicular Injury in Pneumonia.

Study of the data presented fails to reveal any special change characteristic of pneumonia that is produced by a particular organism or that follows any antecedent infectious disease or sequel thereto. Essentially the spermatogenetic function is inhibited and the spermatogonia cease their mitotic activity. They apparently become quiescent while the spermatocytes and other derived cells continue for a time under the influence of the mitotic activation received. This activity is only temporary, for definite signs of degeneration soon appear.

At the same time that nuclear and protoplasmic changes are occurring in the derived cells, the protoplasm of the Sertoli cells is undergoing transformation. It begins to break up, loosening the cells held in its syncytium-like meshes with the result that desquamation is very extensive. Some cells still remain attached by protoplasmic threads, and the free edge of the germinal epithelium appears ragged and frayed. The clusters of spermatids about the Sertoli cells are broken up so that imperfectly formed spermatozoa are added to the collection of cellular debris. Fragmentation of the epithelium and disintegration of all dislodged cells proceed fairly rapidly until the tubule is reduced to a condition similar to that found in childhood and cryptorchidism, in which only the spermatogonia and Sertoli cells remain (Fig. 1). In a few instances the spermatogonia also were caused to disappear, leaving the Sertoli cells alone. Further stages of cellular reduction are not seen without concomitant change in the basement membrane.

Alteration of the basement membrane may begin quite early in the process. Invariably this involves the inner hyaline layer which increases in thickness, and as this occurs and as the tubules shrink, per-

haps from loss of cells, it is thrown into folds which more or less disturb the relation of the spermatogonia to the wall. A moderate amount of thickening of the hyaline layer, perhaps up to three or four times its original thickness, may accompany cellular changes of the severity mentioned in the preceding paragraph, but more extensive changes have been seen only with greater reduction in the cellular elements.

The Sertoli cells usually show no evidence of marked alteration. The nuclei are frequently wrinkled or shrunken to a triangular shape, and a nucleolus can usually be distinguished. They occupy the second row in the germinal epithelial layer and assume a palisade arrangement as the rows of cells on either side disappear (Fig. 2). The protoplasm has the appearance of a fine interlacing network after all the cells are gone. An unusual form of protoplasmic alteration is illustrated by Figs. 3 and 4.

Spermatogonia present as a rule a uniformly normal appearance. In the extreme cases the nuclei are darker, more dense, and may be either larger or smaller. Occasionally a chromatolyzed nucleus can be seen and vacant spaces here and there may indicate complete destruction. Plication of the hyaline basement membrane frequently disarranges the uniformity of their distribution.

The spermatocytes by reason of their size, number, and general prominence exhibit distinct signs of injury. The coiled and interlacing chromatin threads tend to become granular and to break up into coarse darkly stained fragments. These have a tendency to fuse but gradually deteriorate, lose their deep color, and soon appear as mere clusters of granules, perhaps for a time presenting a crescentic appearance, but eventually disappearing entirely. These cells are often too deeply buried in the epithelial wall to break loose or to form any considerable part of the central mass of cellular debris. They are almost invariably decreased and in some instances completely absent, affording convincing evidence that the supply of new cells was cut off and that those already formed are in the process of dissolution.

Spermatids are seen in about half the sections, but are not present in half the tubules of any one section. All are loosened and evidently undergoing degeneration. There have been swelling and fusion of the chromatin, reducing the nuclei to homogeneous deeply stained

masses which are partly clinging to the walls and partly mingled with other loosened cells.

The spermatozoa are quite resistant and are relatively slow in disappearing. In most instances the head has retained its characteristic shape but no tails are to be seen. They are usually widely scattered and have not retained the normal relation to the Sertoli cells as seen during the period of their development.

The desquamated cells present the widest variety of forms. Figs. 5 and 6 give a better idea of this than can be gained from detailed descriptions. The size of many of these cells is surprising, and the presence of more than one pycnotic nucleus in many of them suggests an incomplete separation of the protoplasm, or else the assumption of a globular form by multinucleate portions of the epithelial wall. The nuclei correspond in size and shape to spermatids in most instances. Giant cells which frequently occur here will be described more fully later. Although larger nuclei may represent incompletely divided ones, various forms of caryorrhexis are seen, some involving nuclei larger than any still attached to the wall. The fact that these processes begin while the cells form a part of the epithelium is shown in the drawings. The protoplasm of all these cells is practically homogeneous, contains occasional vacuoles, and has an increased affinity for eosin. Hyaline globules taking a fainter red stain make up a small part of this mass, and their origin in the wall is shown in Fig. 5. They occur normally to about the same extent.

The epithelial layer as a whole is occasionally loosened from the basement membrane, but in the absence of definite evidence to the contrary this condition is regarded as an artifact. The focal nature of the process is strongly suggested in many sections by the varying intensity of the injury to the tubules, thus corresponding to the action of infections upon other organs.

Slight changes in the basement membrane may be due in some cases to apparent thickening which follows an attempt to conform to a tubule of diminished size. More extensive alteration than this is observed in some instances.

The interstitial tissue is subject to considerable variation, some sections showing no increase while others are fibrosed quite irregularly. Occasional areas of round cell infiltration are to be seen about the smaller blood vessels.

The cells of Leydig are generally unchanged, but in some instances they appear to be slightly increased. Such a change might be expected in association with destruction of tubules, but no mitotic figures are to be seen and constructive tissue activity in the presence of a fatal infection is most unlikely. If present the increase must be attributed to previous activity.

In the older, more advanced stages the hyaline membrane continues to increase in thickness with a corresponding decrease in the epithelial cells. A few fibroblasts appear on the inner edge of the membrane and persist until all the Sertoli cells have disappeared. The tubule is now less than half its original size, its lumen is obliterated, and its walls are represented by wavy acellular hyaline material. The size is further diminished and the hyaline material gradually merges with the invading fibroblasts until the tubule is completely lost in the irregular bands of connective tissue that traverse the testes and characterize the condition called fibrous orchitis. The last traces of the tubules are indicated by the suggestive arrangement of the fibroblast nuclei in the periphery, but eventually these too become indistinguishable.

There is no reason to think that any particular part of the process is peculiar to pneumonia or definitely assignable to it. On the contrary, there is every reason to believe that the testicular change found at the death of a pneumonia patient depends chiefly upon the time that the disease has been operative as measured by the number of days in the hospital. Other factors doubtless influence the picture, such as the intensity of the infection, the predominance of certain organisms, etc., but unquestionably the most potent factor is the length of the illness.

In the tables the cases have been so grouped that Table I includes the cases of primary pneumonia that had no antecedent infectious disease, while Tables II and III describe those following measles and epidemic influenza.

Cases of Pneumonia in Which Streptococcus hæmolyticus Occurs.

*Primary Infections (Table I, A).—*There are eleven cases in this series, in which the hemolytic streptococcus alone was found in eight, and associated with other organisms in three. The lobar type of

TABLE I.

Autopsy No.	Length of time in hospital.	Associated organisms.	Principal anatomical diagnosis.	Sertoli cells.	Spermatogonia.	Spermatocytes.	Spermatids.	Spermatozoa.	Desquamated cells.	Giant cells.	Basement membrane.	Interstitial tissue.	Edema.	Older lesions.
A. <i>Streptococcus hemolyticus</i> , occurring in a primary pneumonia, not following an epidemic disease.														
1. Streptococcus alone.														
	days													
53	33		Pulmonary abscesses; empyema.	Norm.	Norm.	Few.	—	—	—	—	Sl. thick.	Incr.	—	—
56	28		Lobular.	Decr.	Decr.	"	—	—	—	—	Thick; hyaline.	" *	+	+
57	36		Atelectasis; empyema, pericarditis.	Norm.	Norm.	"	—	—	—	—	Thick; hyaline.	" *	—	+
58	55		IBP; lobular; empyema.	"	"	"	—	—	—	—	Thick.	"	—	—
171	32		" empyema; acute nephritis.	Decr.	Decr.	—	—	—	—	—	Sl. thick.	Sl. incr.	—	—
174	32		IBP; empyema.	Norm.	Norm.	—	—	—	Rare.	—	"	Norm.	+	—
192	8	St.	Lobular; ulcerative laryngitis.	"	"	Decr.	Decr.	Few.	Many.	Few.	Norm.	"	+	—
193	14	SNh.	IBP.	"	"	"	Few.	Rare.	Few.	Rare.	Sl. hyaline.	Sl. incr.	—	—
2. With <i>B. influenza</i> .														
191	31		IBP; empyema.	Norm.	Norm.	Few.	—	—	Few.	Rare.	Sl. thick; hyaline.	Incr.	—	+
3. With pneumococcus.														
185	3(?)		Lobar; bronchitis; meningitis; pericarditis.	Norm.	Norm.	Decr.	—	—	Few.	—	Sl. thick.	Sl. incr.	—	—

4. With *B. influenzae* and pneumococcus.

186	12	Lobar; empyema; pericarditis.	Norm.	Decr.	Few.	—	Some.	Few.	Sl. thick.	Incr.	—	+
-----	----	-------------------------------	-------	-------	------	---	-------	------	------------	-------	---	---

B. B. influenzae, occurring in a primary pneumonia, not following an epidemic disease.

1. *B. influenzae* alone. No cases.

2. With *Streptococcus hemolyticus*. Identical with A 2 above.

3. With pneumococcus.

51	18	Lobar; peritonitis.	Decr.	Decr.	Few.	—	Some.	—	Norm.	Norm.	++	—
188	8	" acute nephritis.	"	"	"	—	Few.	Rare.	Sl. thick.	Much incr.	—	+

4. With *Streptococcus hemolyticus* and pneumococcus. Identical with A 4 above.

C. Pneumococcus, occurring in a primary pneumonia, not following an epidemic disease.

1. Pneumococcus alone.

19	5	Lobar; empyema; pericarditis.	Norm.	Decr.	Decr.	Few.	—	Sl. hyaline.	Incr.*	—	?
25	7	"	"	"	"	"	—	"	Sl. incr.	—	—

2. With *Streptococcus hemolyticus*. Identical with A 3 above.

3. With *B. influenzae*. Identical with B 3 above.

4. With *Streptococcus hemolyticus* and *B. influenzae*. Identical with A 4 and B 4 above.

IBP has been used for interstitial bronchopneumonia, CLP for confluent lobular pneumonia, and BP for bronchopneumonia. — indicates absence and + presence, while an asterisk denotes the occurrence of small areas of round cell infiltration in the interstitial tissue. In explanation of the meaning of "some" in this connection, it might be said that this is arbitrarily taken as the equivalent of the number of the spermatogonia in any given tubule and hence the terms "many," "few," and "rare" have correlated significance. Norm. indicates normal; decr., decreased; incr., increased; sl., slight or slightly. St. means staphylococcus; StA, *Staphylococcus aureus*; BMC, *B. mucosus capsulatus*; TB, *B. tuberculosis*; Gas B, gas bacillus; SNh, non-hemolytic streptococcus.

lung involvement was found in two cases and five had interstitial bronchopneumonia, while nearly all had empyema or some complication of an infectious nature.

The men were not debilitated by antecedent disease and hence the course of the pneumonia was longer. If the pneumonia is more important in the causation of the testicular changes than an antecedent disease, then these cases of primary infections should show more extensive lesions. It is fair to assume that a septicemia of severe grade and perhaps long duration has been present in all the cases, and in this event the organisms have had abundant opportunity to affect the different parts of the body in an injurious way. That this was true is evident from the extent to which the testes were injured. The Sertoli cells, which have been found to be the most resistant of all the cells in other forms of testicular lesions, suffered evident injury in two cases, and the derived cells were absent in all but three. These instances of extensive and fundamental injury were well distributed over the four subgroups divided according to the presence and kind of associated organisms, and the impression is therefore gained that the presence of other organisms is of no special significance as determining at least this part of the pathology of primary pneumonia.

The spermatogonia were injured and decreased in the same cases in which the Sertoli cells suffered. Spermatozoa were absent in every case but two, and in these they were represented by mere traces. In no instance were they normal in structure, but represented the last stages before complete removal. In one of these cases, No. 193, the section of the seminal vesicle shows that these bodies had been active there until shortly before death.

The microscopic picture presented by the sections was most varied. Nearly all the types of lesions were represented by at least one case, and three was the largest number of instances in which a single type occurred. Acute lesions were found in eight cases, and the more chronic or older lesions occurred in four. This is to be correlated with the fact that very old lesions occur in four instances, that the interstitial tissue is definitely increased in nine, and that two of these have areas of round cell infiltration.

It is perhaps significant to note that ten out of the eleven had definite changes in the thickness and character of the basement membrane.

This has been found to indicate quite clearly the amount of fundamental injury done and to be one of the essential features of the lesions associated with complete atrophy, hyalinosis, and gradual obliteration of the tubules. It is reasonable, therefore, to assume that its initial stages are of some significance, inasmuch as there is no reason to suppose that hyaline changes once begun can be removed with complete restitution of functional integrity.

Streptococcus hæmolyticus Infections Following Measles (Table II, A).—The length of hospital treatment as compared with that in the previous table does not seem to be greater, in spite of the known antecedent disease. In the series of eighteen cases only three had lobar involvement, in two of which the pneumococcus was associated; six had various forms of lobular consolidation, and twelve had the form known as interstitial bronchopneumonia. The last finding is the more surprising in view of the conclusion based upon histological grounds that this represents a tissue-resistant type of disease and the fact that measles has the ability to lower greatly the resistance of the body. This may be the explanation of the relatively shorter period of illness, in that the terminal lung infection caused the death of the patient much more quickly when the body resistance had been previously reduced. If this explanation is true, then the shorter exposure to the effects of the streptococcus would have resulted in less extensive changes in the tubule epithelium. This may be borne out by the fact that only one section showed evidence of injury to the Sertoli cells and two to the spermatogonia. The acute nature of the injury operating over a short period of time is expressed in the finding that all the sections but two contained varying numbers of loose cells in the lumen of the tubules and that there had not been time enough after the injury for them to be completely removed.

On the other hand, the action of the measles in inhibiting the production of spermatozoa is indicated by the fact that they were absent in every section except one. The interpretation of the series is that an influence inhibiting spermatogenesis to a moderate degree had been operative during a sufficient period for spermatozoa already formed to be removed, and that a more acute injurious process had supervened, loosening many of the remaining cells and causing death before they too could be removed. The lesions were diversified, the

TABLE II.

Autopsy No.	Length of time in hospital.	Associated organisms.	Principal anatomical diagnosis.	Sertoli cells.	Spermatogonia.	Spermatozoa.	Desquamated cells.	Giant cells.	Basement membrane.	Interstitial tissue.	Edema.	Older lesions.
A. <i>Streptococcus hemolyticus</i> , occurring in a pneumonia following measles.												
1. <i>Streptococcus</i> alone.												
days												
206(?)			Lobular; empyema.	Norm.	Norm.	Many.	Many.	—	Norm.	Norm.	—	—
34 14			IBP; empyema.	"	"	Few.	Few.	—	Sl. hyaline.	Incr.*	—	—
41 17			"	"	"	"	Some.	Rare.	" thick.	"	+	+
50 36			" lobar; empyema.	"	"	"	"	"	" hyaline.	Sl. incr.	+	+
55 25			Lobular; septicemia; empyema.	Decr.	Few.	—	—	—	" thick.	Norm.	+	+
176 45			IBP; empyema; pericarditis; abscesses.	Norm.	Norm.	—	Few.	—	Norm.	"	+	—
178 17	St.A.		IBP; ulcerative laryngitis.	"	"	—	Some.	Rare.	Sl. thick.	Sl. incr.	—	+
184 34			"	"	"	Few.	Many.	Few.	"	Incr.	—	—
194 13			" lobular; empyema; ulcerative laryngitis.	"	"	—	Some.	"	"	Sl. incr.	—	—
195			Lobular; empyema.	"	"	Few.	Many.	"	"	"	+	—
204 11			IBP, with abscesses; empyema; pericarditis.	"	"	—	Few.	—	"	"	+	—
2. With <i>B. influenzae</i> .												
327(?)			IBP.	Norm.	Norm.	—	Few.	—	Norm.	Sl. incr.*	+	+
37 16			" empyema.	"	"	Decr.	Some.	Few.	"	Norm.	+	—
202 21			BP; "	"	Decr.	—	Few.	—	Sl. thick.	Incr.	—	+
205 25			IBP; lobular; empyema; otitis media.	"	Norm.	Few.	"	Rare.	"	"	—	?

3. With pneumococcus.											
31	25	IBP; lobar; empyema. Lobar; empyema; pericarditis.	Norm. "	Few. Decr.	— Few.	— —	Few. Some.	— Rare.	Sl. hyaline. " thick.	Sl. incr.* Incr.	+ —
46	5										
4. With <i>B. influenzae</i> and pneumococcus.											
33	35	Lobar; empyema.	Norm.	Few.	—	—	—	—	Sl. thick.	Sl. incr.	+ —
B. <i>B. influenzae</i> , occurring in a pneumonia following measles.											
1. <i>B. influenzae</i> alone. No cases.											
2. With <i>Streptococcus hemolyticus</i> . Identical with A 2 above.											
3. With pneumococcus. No cases.											
4. With <i>Streptococcus hemolyticus</i> and pneumococcus. Identical with A 4 above.											
C. Pneumococcus, occurring in a pneumonia following measles.											
1. Pneumococcus alone. No cases.											
2. With <i>Streptococcus hemolyticus</i> . Identical with A 3 above.											
3. With <i>B. influenzae</i> . No cases.											
4. With <i>Streptococcus hemolyticus</i> and <i>B. influenzae</i> . Identical with A 4 and B 4 above.											

TABLE III.

Autopsy No.	Length of time in hospital.	Associated organisms.	Principal anatomical diagnosis.	Sertoli cells.	Spermatogonia.	Spermatocytes.	Spermatis.	Spermatzoa.	Desquamated cells.	Giant cells.	Basement membrane.	Interstitial tissue.	Edema.	Older lesions.
A. <i>Streptococcus hemolyticus</i> , occurring in a pneumonia following epidemic influenza.														
1. Streptococcus alone.														
	days													
219	10		CLP; pleurisy.	Norm.	Norm.	Decr.	Few.	Few.	Some.	Few.	Norm.	Sl. incr.	—	—
2. With <i>B. influenzae</i> .														
89	24		IBP; pericarditis.	Norm.	Norm.	Few.	Few.	—	Few.	—	Sl. thick.	Incr.	—	+
3. With pneumococcus.														
226		St.	CLP; pleurisy.	Norm.	Norm.	Norm.	Decr.	Few.	Some.	Rare.	Norm.	Sl. incr.	—	?
4. With <i>B. influenzae</i> and pneumococcus.														
83	11	St.	CLP; pleurisy.	Norm.	Norm.	Decr.	Decr.	Some.	Few.	Rare.	Norm.	Norm.	—	—
88	15	StA.	IBP; " myocarditis.	"	"	Few.	Few.	—	"	—	"	"	—	—
B. <i>B. influenzae</i> , occurring in a pneumonia following epidemic influenza.														
1. <i>B. influenzae</i> alone.														
81	19		IBP; pleurisy.	Norm.	Norm.	Few.	Few.	—	Some.	Rare.	Norm.	Incr.*	—	—
86	21		" " endocarditis.	"	"	"	—	—	Few.	—	Sl. thick.	Sl. incr.*	—	?
211	9	StA.	CLP.	"	"	Decr.	Decr.	Some.	Some.	Few.	" hyaline.	Norm.	+	—
2. With <i>Streptococcus hemolyticus</i> . Identical with A 2 above.														
3. With pneumococcus.														
80	15	St.	CLP; pulmonary embolism.	Norm.	Norm.	Decr.	Decr.	Decr.	Few.	Rare.	Sl. thick.	Sl. incr.	—	—
82	7	"	" pleurisy; ulcerative laryngitis.	"	"	"	"	Some.	Some.	Few.	" hyaline.	"	—	—

84	11 St.	CLP; ulcerative laryngitis; endocarditis.	Norm.	Norm.	Few.	—	—	Some.	Rare.	Norm.	Sl. incr.	+
85	10 BMC.	CLP; jaundice.	"	"	"	—	—	Few.	"	Sl. thick; hyaline.	Incr.	+
87	17	"	"	"	—	—	—	—	—	Thick; hyaline.	"	++
90	23 TB.	IBP, with pulmonary abscesses; empyema; endocarditis. Lobular; tuberculous cavities.	"	"	Few.	—	—	Few.	Rare.	Sl. thick; hyaline.	" *	+
214	13	CLP.	"	"	Decr.	Few.	Few.	Many.	Some.	Sl. hyaline.	"	+
215	10 St.	" pleurisy; ulcerative laryngitis.	"	"	"	"	"	Few.	Rare.	Norm.	Sl. incr.	—
216	15	CLP; pleurisy.	"	"	"	"	"	"	"	Sl. thick.	Incr.	+

4. With *Streptococcus hemolyticus* and pneumococcus. Identical with A 4 above.

C. Pneumococcus, occurring in a pneumonia following epidemic influenza.

1. Pneumococcus alone.

212	19	CLP; pleurisy.	Norm.	Norm.	Decr.	Some.	Some.	Few.	Rare.	Norm.	Norm.	+
217	23	" " emphysema (Gas B).	"	"	"	"	—	—	Few.	Sl. hyaline.	Incr.	+
220	15 TB.	CLP; pleurisy; tubercles in spleen.	"	"	"	"	Few.	Many.	"	" thick.	"	+
221	11	CLP; pleurisy.	"	"	"	"	"	Some.	"	Norm.	"	?
222	"	"	"	"	Norm.	Some.	"	"	"	Sl. hyaline.	Sl. incr.	—
223	9	"	"	"	Decr.	Decr.	"	"	Rare.	"	"	—
224	9	"	"	"	"	"	"	Few.	"	Norm.	Incr.	—
227	"	"	"	"	"	"	Many.	Some.	Few.	"	Sl. incr.	?
228	12	"	"	"	Few.	"	—	Few.	Rare.	Sl. thick.	"	—
229	10	" " mitral stenosis.	"	"	Decr.	Decr.	Few.	"	"	" hyaline.	"	—

2. With *Streptococcus hemolyticus*. Identical with A 3 above.

3. With *B. influenza*. Identical with B 3 above.

4. With *Streptococcus hemolyticus* and *B. influenza*. Identical with A 4 and B 4 above.

type in Fig. 6 being found in six sections. The chronic types of lesions appeared in seven cases, while evidence of acute change was seen in sixteen. Very old lesions were seen in eight slides, and those of a doubtful character in still another. Interstitial tissue increase affected all but four cases. Moreover, the basement membrane was thickened in all except four. Round cell infiltration appeared in three cases and the so called edema in nine.

No obvious difference can be made out between the cases in which the streptococcus occurred alone and those in which it was associated with one or more pathogenic forms.

Streptococcus hæmolyticus Infections Following Epidemic Influenza (Table III, A).—The type of lung lesion is still different in these cases, there being no lobar consolidations, and only two cases of interstitial bronchopneumonia. The remaining three were diagnosed confluent bronchopneumonia, a type of lung lesion supposed to be low in the scale of resistance. Pleurisy accompanied nearly all these cases and empyema was completely absent. The pneumonia was the terminal condition in every instance, and nothing of a more prolonging nature occurred as a complication or sequel. The period of illness incident to the influenza was as usual short, and the pneumonia following was also of short duration. As a result the stay in the hospital was from 10 to 24 days, averaging a much shorter time than in the two preceding series. Subdivision of these cases gives a definitely longer period of illness for those having interstitial pneumonia than those with the confluent type.

The microscopic findings correspond closely with this picture. The Sertoli cells and spermatogonia have been apparently uninjured. The spermatocytes and spermatids have been much reduced in all cases but not completely removed in any, and the spermatozoa are still present in three out of five. Loosened cells occur in all, and the basement membrane has been thickened only in the case that lived the longest. The whole picture indicates clearly that the preceding influenza had little effect in the inhibition of spermatogenesis, possibly because of the short time in which it could manifest itself and because the pneumonia that followed was so fulminant in character that the patient died before the cells of the tubules were more than loosened from their attachments.

Cases of Pneumonia in Which Bacillus influenza Occurs.

*Primary Infections (Table I, B).—*These cases developed during the measles epidemic, but so far as could be learned they had not had that disease. There were no instances in which the organism occurred alone, and those in which the streptococcus was found have been previously described. In three of the four cases the lobar type of lung involvement was found, and one had interstitial bronchopneumonia. The two cases in which the pneumococcus was also found are apparently more severely injured than that associated with the streptococcus; however, the case affected by all three organisms manifested the least injury. There was a definite lack of uniformity about the picture presented by this group, suggesting that although it belonged to a supposedly primary infection, the associated organisms were perhaps more potent than the one in common. This fact suggests that *Bacillus influenza* is not of importance as a cause of testicular change.

*Bacillus influenza Infections Following Measles (Table II, B).—*There were no cases in which *Bacillus influenza* was found alone or associated with the pneumococcus. The entire five cases were associated with the streptococcus alone or with the latter and the pneumococcus. The conclusion is therefore readily drawn that the streptococcus is the dominant organism, and a discussion of its effects here would be unwarranted.

*Bacillus influenza Following Epidemic Influenza (Table III, B).—*Three cases are reported in which this organism occurred alone, one with the streptococcus, nine with the pneumococcus, and two with both. The cases with the streptococcus or without other organisms were usually of the interstitial bronchial type, while those in which the pneumococcus was found were largely of the confluent lobular variety. One case had also small tuberculous cavities. Prolonging complications were absent and the longest period of illness was 24 days. Possibly the shortness of the time has influenced the relative uniformity of the picture inasmuch as the various groups have no observable differences. The spermatogonia and Sertoli cells were not obviously affected in any case. The interstitial tissue of the sections was increased in two. Perhaps this was just a coincidence, as it is hard to understand how an acute infection could have produced such a result. The presence of old lesions accompanied this increase in most instances.

Cases of Pneumonia in Which the Pneumococcus Occurs.

The cases of pneumonia as they occurred among the civil population under pre-war conditions have been considered to be caused chiefly by the pneumococcus. Occasional mention of *Streptococcus hemolyticus*, and more rarely of the influenza bacillus, has been made, but on bacteriological and serological grounds the pneumococcus has been regarded as the chief etiological factor. Doubtless the attention that has been given the other forms during the present epidemics will serve to emphasize their importance and lead to their recognition in the future in a larger percentage of cases. It remains to be seen how long they will retain their activity in the causation of pneumonia after camp concentration has ceased, and a satisfactory determination of this point will enable one to review these cases and to pass more mature judgment than is now possible. The impression seems to be that the original importance of the pneumococcus in the production of pneumonia was, at least during the measles epidemic, superseded by *Bacillus influenzae* or *Streptococcus hemolyticus* or both these organisms. The cases described by Cordes were doubtless similar to other pre-war cases and were not even comparable from an etiological standpoint with any of the present series of cases. The latter, it must be remembered, were probably caused by the same organisms that produced the pneumonia directly following the epidemic diseases, and were perhaps more virulent than usual, an idea strongly supported by the extraordinary incidence of pneumonia at that time.

Primary Infections (Table I, C).—Six cases are collected and in all the lobar type of consolidation was found. Several had complications such as empyema, peritonitis, and pericarditis, but most of them terminated after short periods of illness. Two cases were found at the beginning of the measles epidemic, in which the pneumococcus occurred alone, all the later ones being complicated by the presence of the streptococcus and the influenza bacillus.

The type of pneumococcus was not uniform, all the types being represented in about the same proportions.

The pathological picture of the cases with only the pneumococcus present was relatively simple and uniform. Spermatogenesis was not completely stopped, as evidenced by the presence of at least a few

spermatozoa and only a moderate degree of reduction in the derived cells. The basement membrane was only slightly hyalinized and the interstitial tissue was slightly increased. All but one of these six cases had thickened basement membranes and in nearly all the interstitial tissue was increased. The one without changes in the basement membrane or increase in the interstitial tissue had a pronounced separation of the tubules which has been described as edema.

Pneumococcus Infections Following Measles (Table II, C).—There were no cases in which this occurred alone or in conjunction with *Bacillus influenzae*. The three cases enumerated were all associated with the streptococcus, alone or in conjunction with *Bacillus influenzae*. The importance of the streptococcus in this group of postmeasles cases is emphasized by the relative unimportance of the pneumococcus and the influenza bacillus. The details of the picture have been sufficiently discussed above.

Pneumococcus Infections Following Epidemic Influenza (Table III, C).—The influence of the pneumococcus seems to be relatively greater in this series than it was in those preceding. There were ten cases in which this was the only organism found, one in which it was associated with the streptococcus, nine with *Bacillus influenzae*, and two with both. Those with a single infection were uniform in the general disease picture, having simply confluent lobular pneumonia and pleurisy. In the other groups in which other organisms perhaps played a part the same general features were repeated with little variation.

The Sertoli cells and spermatogonia were unaffected, and only moderate degrees of injury to the derived cells were seen. Spermatozoa were still present in all but seven cases, and spermatids persisted in all but five. The types of lesion in almost every instance corresponded to those in Fig. 5.

DISCUSSION.

Presumably there exists a period following the onset of an infection, if this is rather sudden and acute, in which the testes show little evidence of injury. Somewhat analogous conditions are found in early death from mercuric chloride poisoning in which the kidneys have had little time to give visible evidence of the chemical injury done. Such an overwhelming bacterial intoxication is not seen in pneumonia, and

hence this prereactionary stage is not represented in the series. From febrile changes in other organs it would seem that these morphological alterations should begin to appear rather early and hence would be well under way within the period of an ordinary pneumonia.

The separation of the cells in the epithelial wall somewhere central to the row of Sertoli cell nuclei, which is an occasional finding, causes a loosening of the spermatids and of some of the spermatocytes. If the Sertoli cells receive nourishment from the cells of Leydig, as is commonly believed, it is reasonable to suppose that protoplasmic changes might early affect the nutrition of the derived cells. Hanes (7) believes that one function of the Sertoli cells is to supply fat to the developing sperm cells, and hence it may be assumed that any interruption of this process would lead to fragmentation of the protoplasm with loosening of these cells.

If, on the other hand, the toxic action is slower and more continuous, the same result would appear in a more insidious and gradual manner. This is really more nearly according to facts, in that a fragility exists which leads to breaks in the continuity.

It is conceivable that mitotic activity of sperm cells would be inhibited in much the same way that other functions of epithelium are reduced in fevers; for example, the secretion of the sebaceous glands. These would naturally resume activity if recovery from the initial disease resulted, but in fatal cases would show no tendency toward restoration.

However, inhibition of spermatogenesis is not always a pathological process. Investigations of Hanseemann (8) on hibernating and waking marmots show that this change occurs normally and that restoration consists of the formation of a new supply of derived cells by multiplication of the spermatogonia that have been for a time quiescent. Grandis (9) found that spermatogenesis ceased in fasting doves after a few days; the spermatozoa already formed were speedily removed and most of the cells in the tubules died. The detritus appeared to be diverted for the nourishment of the bird but the spermatogonia persisted and formed new elements after the hunger was relieved. There is no reason, therefore, for regarding the mere death and removal of the derived cells as unusual, when we consider the metabolic drain imposed upon the system by an acute and prostrating fever. Injury to

the spermatogonia and perhaps changes in the supporting framework are, however, matters of special concern. The method by which the damage is accomplished and the variations in the appearance of the products of degeneration are worthy of study as possible indices of the cause of the injury and a measure of its power.

Theoretically if the infection is responsible for the testicular injury, the greatest degree of alteration would be shown in the lists of cases in which that agent was the common factor. Variations might occur in the severity in proportion to the virulence of the associated organisms or prominence of other pathological conditions. Uniformity of results in any one table, then, would strongly suggest the paramount potency of the one or two common factors.

However, to differentiate the true lesions of pneumonia from the changes in the testes properly attributable to autolysis and postmortem alteration is not always easy. Most of the autopsies in this series were done shortly after death in order to secure cultures of organisms as free as possible from contaminations. It is therefore unlikely that more than 3 or 4 hours elapsed before the tissues were placed in fixing fluid.

The suggestion has been made that the separation of the epithelium from the basement membrane and the consolidation of the former in the interior with loss of lumen are not a technical fault, but evidence of a serious injury that leads to atrophy. Such distortions are common in various poorly fixed or greatly shrunken tissues and hence such an interpretation here would not be unwarranted. On the other hand, in sections in which there has been little or no cellular decrease and those from accident cases in which infection and other morbid influences had little time to act, this condition was absent. Very rarely are healthy spermatozoa and shrunken tubules found in the same section.

A condition resembling edema has been portrayed in Figs. 7 and 8, and has been described before. Councilman, Magrath, and Brinckerhoff (10) mention an edema of the interstitial tissue in smallpox in relation to similar epithelial changes in the testes. In pneumonia an acute alteration in the tubules, often indicated by the coexistence of a definite shrinking of the epithelial masses and a rarefaction of the interstitial tissue, has been described by Weichselbaum and Kyrle

as edema. This coincidence exaggerates the epithelial change and is quite striking. In these spaces, however, granules of coagulated protein are usually lacking.

The interstitial tissue is also subject to increase in density. This may be a diffuse process or may take the form of irregular bands of connective tissue, more or less associated with hyalinized tubules. The blood vessels are thickened in some instances. Weichselbaum and Kyrle consider that the desquamation of cells is a part of the pathological picture, whereas Cordes believes it to be a postmortem phenomenon. This series of cases points definitely to the correctness of the former statement. It seems clear that the number of desquamated cells progressively decreases after the initial injury, and that, other things being equal, the number of loosened or injured cells remaining is an index of the length of this period.

The presence of giant cells is one of the structural features that call attention to the abnormality of the process. They have been described before in a variety of testicular lesions, but until recently no explanation of their origin was offered. Weller (11) apparently correctly considers them to be the result of attempts at cell division in which the separation of the protoplasm is delayed. The nuclei are usually peripherally arranged and may surround a vacuole. The size of the nuclei is variable, some corresponding to those of spermatids and others being somewhat smaller. The nuclei number from two to six or more and correspond structurally with the spermatids in which pycnosis has taken place.

The point of formation is definitely shown in Fig. 5, in which a fully developed giant cell is still attached in the zone of the spermatids. It is hardly likely that further development takes place after desquamation. No mitotic figures have been seen in them and no evidence of phagocytosis. The protoplasm stains uniformly but heavily with eosin, and the cell aggregate has assumed a circular form, doubtless as a result of cohesive force. The number of these giant cells is variable. They are most numerous where the amount of desquamated material is greatest, but rapidly diminish as the other cells disappear, being among the first to go. They are more abundant in the simplest cases of postinfluenzal pneumonia where the number of desquamated cells is greatest.

SUMMARY.

The testicular changes in pneumonia are without clinical manifestations, are non-specific, focal in character, independent of the infecting organisms or the antecedent disease, and vary in severity directly with the total length of the illness. The process is a continuous one, divisible into stages in which the following features are recognizable: (1) cessation of spermatogenesis; (2) degeneration of preformed spermatocytes, spermatids, and spermatozoa; (3) desquamation of altered cells and fragments of the same; (4) formation of giant cells in the tubule walls with subsequent liberation into the lumen; (5) disappearance of all desquamated cells and all those derived from the spermatogonia by mitosis; (6) in some instances thickening of the hyaline layer of the basement membrane.

Older lesions are frequently found which continue the structural alteration of the tubules by hyalinosis and destruction of cells until they ultimately disappear. These lesions are not believed to be connected with the present illness.

Edema may represent the acute injury in another form, and round cell infiltration suggests that possibly other factors than toxins may have a part in the tissue alterations.

In the absence of definite evidence to the contrary, the cause is assumed to be circulating toxins, as Wolbach (12) claims for influenzal cases.

The hemolytic streptococcus produced more extensive changes, both epithelial and interstitial, in primary pneumonia occurring during the measles epidemic than when pneumonia followed as a secondary infection; in the latter cases the pulmonary complications covered a relatively shorter period. Measles and epidemic influenza had little apparent effect upon the testes, except that the former caused mild inhibition of spermatogenesis; evidence regarding the latter is inconclusive.

The Pfeiffer bacillus was always associated with other organisms, in primary infections and in those following measles. It occurred alone in a few cases after epidemic influenza, but the testicular lesion was not distinctive.

The pneumococcus when alone in primary infections or after an epidemic disease produced a uniformly mild picture which was not intensified when associated with the influenza bacillus.

Giant cells were much more frequent after influenzal pneumonia regardless of its cause and were associated with large numbers of other desquamated cells. They are formed in the walls of tubules by futile mitotic effort and incomplete protoplasmic separation, the abnormality of the process being further suggested by the early severing of cytoplasmic attachments and rapid desquamation.

The series is unique in its uniformity, in the care exercised in the bacteriological examinations, and in the relative freedom from complicating factors.

The author is under deep obligation to Professor W. G. MacCallum for material and cordial cooperation.

BIBLIOGRAPHY.

1. Osler, W., and McCrae, T., *Modern medicine*, Philadelphia and New York, 2nd edition, 1913, i.
2. Cordes, H., *Virchows Arch. path. Anat.*, 1898, cli, 402.
3. MacCallum, W. G., *J. Am. Med. Assn.*, 1919, lxxii, 193.
4. MacCallum, W. G., *The pathology of the pneumonia in the United States Army Camps during the winter of 1917-18*, Monograph of The Rockefeller Institute for Medical Research, No. 10, New York, 1919.
5. MacCallum, W. G., *J. Am. Med. Assn.*, 1919, lxxii, 720.
6. Weichselbaum, A., and Kyrle, J., *Sitzungsb. k. Akad. Wissensch. med.-naturw. Kl.*, 1912, cxxi, 51.
7. Hanes, F. M., *J. Exp. Med.*, 1911, xiii, 338.
8. Hansemann, D., *Virchows Arch. path. Anat.*, 1895, cxlii, 538.
9. Grandis, V., *Arch. ital. biol.*, 1889, xii, 215.
10. Councilman, W. T., Magrath, G. B., and Brinckerhoff, W. R., *J. Med. Research*, 1904, xi, 12.
11. Weller, C. V., *Proc. Soc. Exp. Biol. and Med.*, 1916-17, xiv, 14.
12. Wolbach, S. B., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 104.

EXPLANATION OF PLATES.

The illustrations were drawn with a camera lucida at a magnification of 500 diameters. The cells are therefore comparable in size, but for convenience the smallest tubule that would show the lesion was selected.²

² The helpful suggestions of Professor Max Brödel are greatly appreciated.

PLATE 45.

FIG. 1. Case 165. A quiescent stage in which only spermatogonia and Sertoli cells remain. The protoplasm of the latter is merely a fine network, some of the holes in which represent the places from which the derived cells have been removed.

FIG. 2. Case 216. Further reduction with persistence of Sertoli cells only. Shrinkage of the wall probably due to faulty technique.

PLATE 46.

FIG. 3. Case 216. A special form of protoplasmic change in which large deep red granules obscure all traces of cellular structure. Note the relation to adjacent protoplasm suggesting infiltration or expansion and lack of continuity with neighboring cells.

PLATE 47.

FIG. 4. Case 216. A more advanced stage of the process shown in Fig. 3. The nuclei are not materially affected and apparently belong to Sertoli cells. An unaffected tubule is shown in the upper left corner and the mass of connective tissue on the right is part of a heavy band containing remnants of destroyed tubules, suggesting that this process is definitely destructive.

PLATE 48.

FIG. 5. Case 227. A common picture in postinfluenzal cases. Large numbers of desquamated cells including giant cells, hyaline bodies, and spermatozoa. Cells with deep red homogeneous protoplasm are breaking away from the free margin and one giant cell is still embedded in the wall.

PLATE 49.

FIG. 6. Case 90. Fewer desquamated cells and greater reduction in the derived cells. The Sertoli cells are quite definite as the second row, and the spermatogonia next to the wall are moderately well preserved. The basement membrane is slightly thickened and thrown into small folds.

PLATE 50.

FIG. 7. Case 51. Great edema of the interstitial tissue with collapse of tubule walls upon the decreased cellular mass. Acute degeneration of all the cells including spermatogonia and Sertoli cells.

FIG. 8. Case 53. Collapse of tubule walls and adjacent tissue upon a tubule in a resting stage, similar to that in Fig. 1. Edema of the interstitial tissue.

570

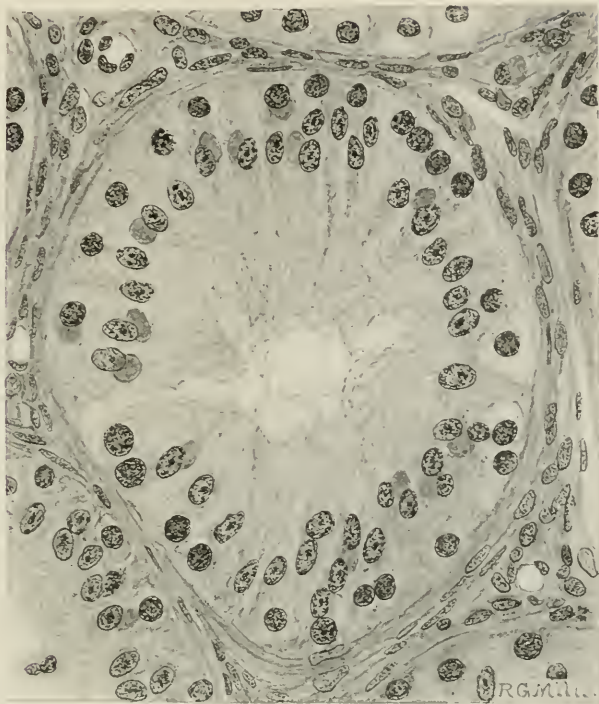


FIG. 1.



FIG. 2.

(Mills: Changes in testes in pneumonia.)

5302

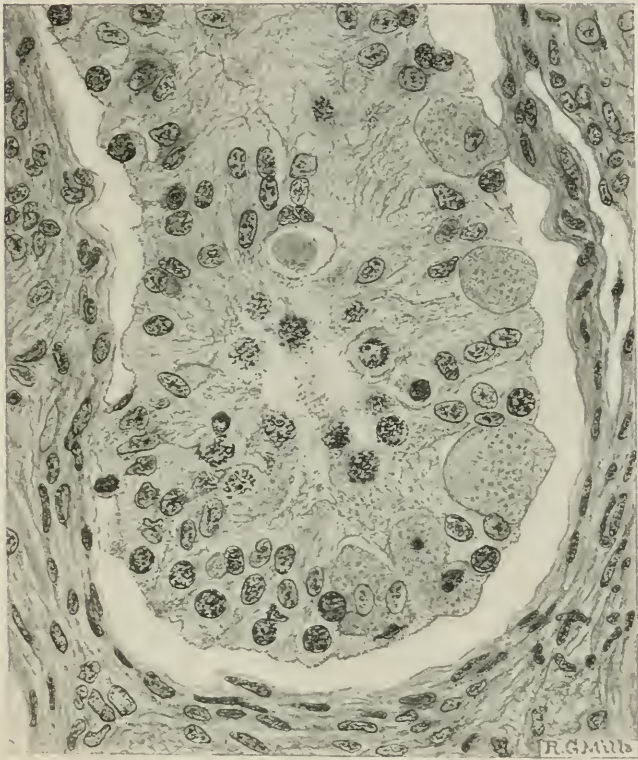


FIG. 3.

(Mills: Changes in testes in pneumonia.)



FIG. 4.

(Mills: Changes in testes in pneumonia.)

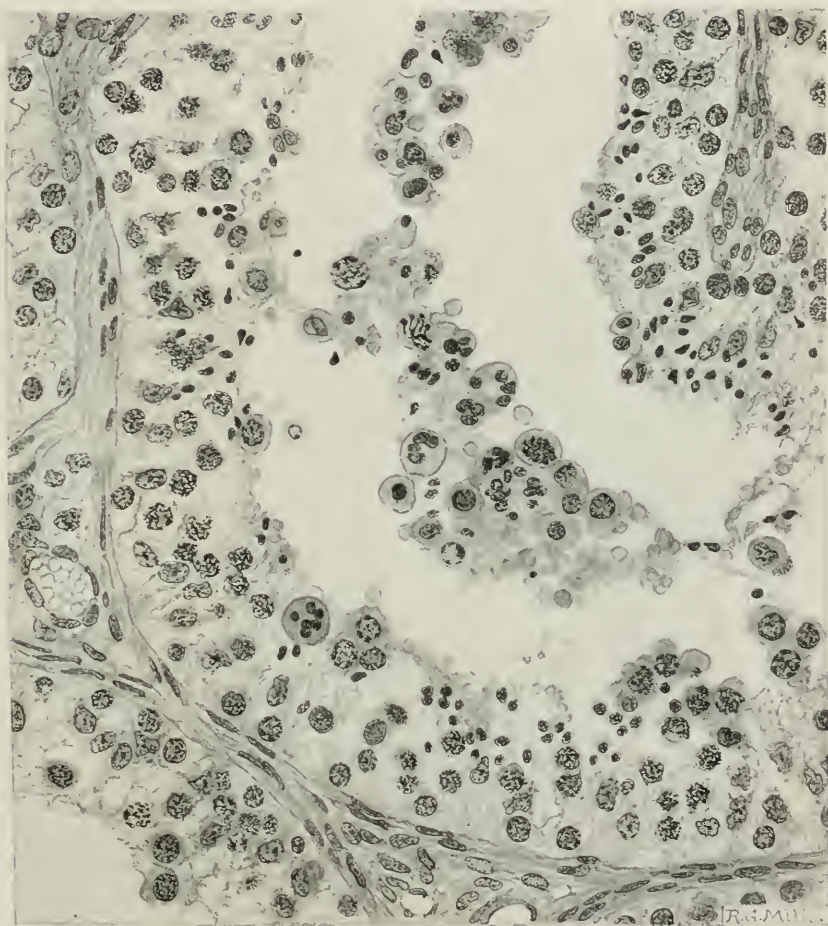


FIG. 5.

(Mills: Changes in testes in pneumonia.)

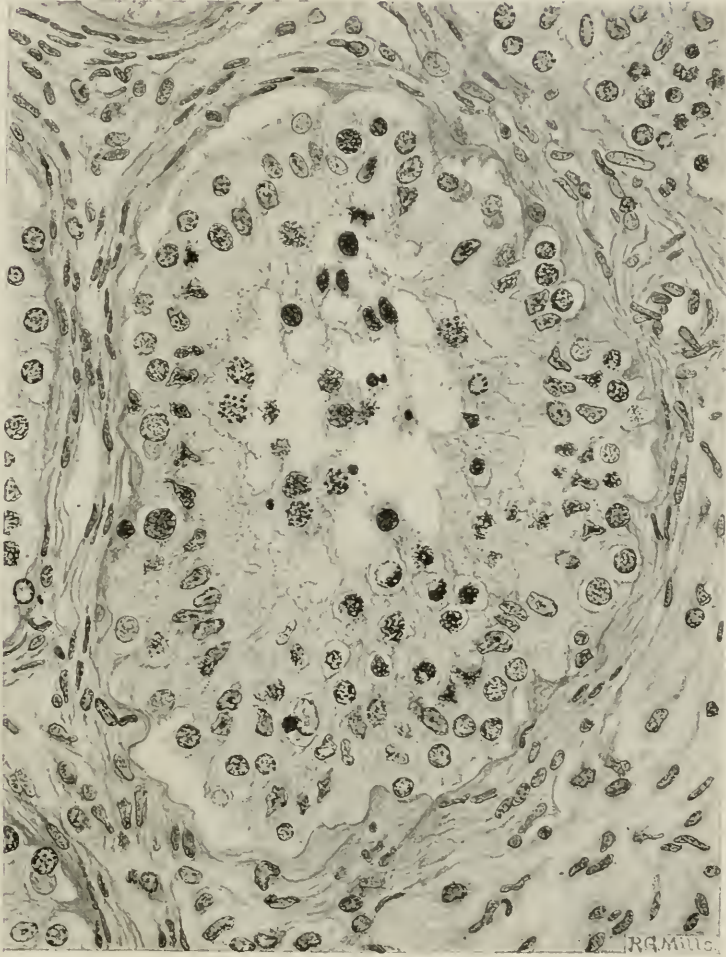


FIG. 6.

(Mills: Changes in testes in pneumonia.)

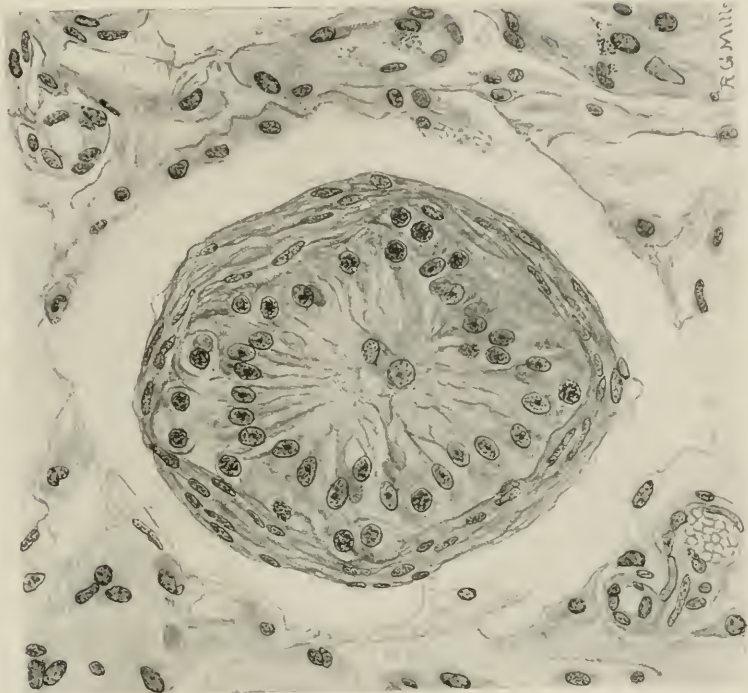


FIG. 8.

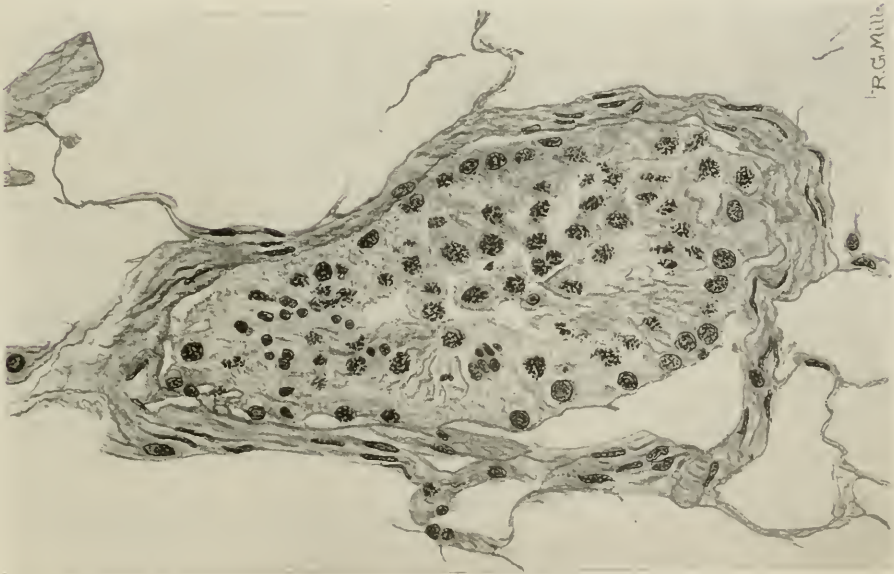


FIG. 7.

(Mills: Changes in testes in pneumonia)

A STRAIN OF CONNECTIVE TISSUE SEVEN YEARS OLD.

By ALBERT H. EBELING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 51 to 55.

(Received for publication, July 7, 1919.)

In July, 1914, Dr. Carrel reported the condition of a strain of connective tissue 28 months old, isolated from a fragment of heart extirpated from a chick embryo on January 17, 1912.¹ Today this strain is still alive. It has been under cultivation *in vitro* for a period of over 7 years and has undergone 1,390 passages.

The purpose of this article is to describe the technique employed in perpetuating the strain during the last 5 years and in measuring the increase of the tissue, the factors which influence the rate of growth, and the present condition of the strain.

Technique.

The technique does not differ fundamentally from the technique already reported.² The fragments of the old strain are allowed to grow undisturbed for 48 hours and are then divided into two parts and transferred to a fresh medium in the following manner.

The cover-glass is lifted with the heated point of a blunt cataract knife. It is then placed upon a piece of black glass. The fragment is extirpated by four clean cuts made with the blade of a sharp cataract knife within the area of new growth, and divided into two or three pieces as equal in size as possible. The pieces are transferred, with the point of the knife and a needle if necessary, to a bath of Ringer's solution. They are allowed to remain in the bath for about 45 seconds. Meanwhile the substances composing the medium are dropped upon a cover-glass and thoroughly mixed with the end of the cataract knife. Then the medium is spread over the surface of the glass, in

¹ Carrel, A., *J. Exp. Med.*, 1914, xx, 1.

² Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273.

order that the depth and area of the clot may be approximately uniform in all preparations.

The fragments of tissue are transferred by means of the knife point to the medium. They must be embedded thoroughly in it, without folding or curling. This step must be carried out rapidly to guard against embedding after coagulation has set in; that is, after 15 to 20 seconds, under ordinary conditions of room temperature and moisture. Coagulation is allowed to proceed and occurs in from 45 to 50 seconds. During this period, as well as during the period of washing in Ringer's solution, the preparations are kept under a large Petri dish in order to eliminate as nearly as possible chance bacterial contamination from the dust of the atmosphere.

After coagulation the cover-glass is inverted and placed on a hollow slide and held in place by a small quantity of vaseline. When coagulation does not occur promptly, the slide is prepared with vaseline in the manner described above, but instead of inverting the cover-glass over the slide, the slide is inverted and placed over the cover-glass, care being exercised not to touch the periphery of the medium. The slide is then picked up with its adherent cover-slip and set aside until coagulation occurs. This procedure prevents undue evaporation, which occurs if the medium is exposed for any length of time to the surrounding air, even though kept under the large Petri dish. The slides are sealed with paraffin melting at 56°C. and placed in the incubator at an average temperature of 39°C.

The medium used for perpetuating the strain is composed of equal volumes of chicken plasma and chick embryo extract. This combination produces a clot firm but not dense enough to interfere with the migration of the cells. Although a mixture of one volume of extract and two volumes of plasma constitutes a satisfactory medium, it has been observed that the new growth is less extensive. The clot is firmer and contracts more closely around the tissue fragment. It seems that the central portion of the tissue is not reached by fresh medium. The cells composing it die, and the necrotic tissue appears to retard the growth of the peripheral portion of the culture.

The plasma is obtained from adult chickens. The blood is taken from the carotid artery through an oiled glass cannula, received in chilled paraffined tubes, and centrifuged. Finally, the supernatant

plasma is pipetted off and kept in tightly corked paraffined tubes in cold storage at 4°C. The best plasma is obtained from young, healthy adult chickens, not over 2 years old, which have not been fed for a period of 24 hours previous to bleeding. Blood withdrawn from more recently fed chickens gives a turbid plasma rich in fat globules. Such plasma gives a hazy clot in which the cells do not develop so well as in a clear plasma.

The tissue extract employed is obtained from 7 to 8 day chick embryos. The embryos are first washed in Ringer's solution to remove the amniotic fluid and traces of blood which adhere to them after removal from the shell. Then the solution is drawn off in order to obtain an undiluted tissue juice. The embryos are minced in a watch-glass with sharp, curved scissors. The pulp is centrifuged for 10 minutes and the supernatant fluid obtained is drawn off.

The area of new growth is measured by means of a projection apparatus. In adjusting the condenser it is essential to guard against the action of the light rays upon the tissues, since the heat developed by concentration of the radiations is injurious. Diffused light sufficiently powerful to give proper illumination without any noticeable ill effect must be used. The image of the fragment of tissue is cast upon a sheet of paper, and by means of a pencil the outline of the original fragment and that of the new tissue can be traced. Shortly after the cultures are prepared, a drawing of the outline of the piece of embedded tissue is made, which requires not more than 20 seconds. The culture can then be returned to the incubator. Subsequent tracings, after growth is well established, are made at intervals on the same sheet of paper. They must be made rapidly because, after a short time, small droplets form and settle in the concavity of the slide. These droplets, usually after a period of from $1\frac{1}{2}$ to 2 minutes, coalesce and then no longer interfere with focusing. Such a long exposure of the tissue to the light has probably a retarding effect on the growth and should be avoided.

The rate of growth is expressed in function of the initial area for a given time interval. That is, the area of the newly grown tissue is equal to n times the initial area, as, for instance, it may be said that it has doubled or tripled, or that it is 10 times larger, within the same interval of time. Then, the rates of growth of two fragments of tissue

of unequal size can easily be compared. For example, the area of a 7 sq. cm. fragment, and of a 5.8 sq. cm. fragment, became in 48 hours, respectively, 99 sq. cm., and 81.5 sq. cm. The first one increased 92 sq. cm., the second only 75.7 sq. cm. However, the relation of the size of the growth to the size of the initial area is identical:

$$\text{No. 1, } \frac{99-7}{7} = 13.1 \qquad \text{No. 2, } \frac{81.5-5.8}{5.8} = 13.1$$

Each fragment has become 13.1 times as large as it was. The relation existing between the rates of growth of two fragments can be expressed in the form of a ratio, which is equal to $\frac{13.1}{13.1} = 1$ in the above example.

Factors Which Influence the Rate of Growth.

The strain is used chiefly for measuring the influence of different factors on the rate of growth of connective tissue. The value of the method depends entirely on the property of the parts of a divided fragment of tissue to grow at the same rate when they are cultivated in identical media. Therefore it is important to know what factors may cause two pieces of the same fragment to grow at unequal rates, and to recognize the presence of these factors after the fragments have been embedded in their medium.

As a rule, when two parts of a fragment of the strain which has been growing actively for 48 hours are cultivated in identical media, they grow at the same rate. However, it was found that parts equal or unequal in size coming from the same original fragment do not always grow at the same rate. The reasons for this difference have been determined in such a manner that examination of the pieces makes it possible almost always to foresee whether they will grow at the same rate, and then to discard the useless cultures.

Fragments identical in area may differ in thickness. This may be seen easily by microscopic examination, and sometimes by the naked eye. When a region whiter than the surrounding tissue is seen, it is probable that it is thicker and possibly necrotic. Microscopic examination of the tissue then shows a spot very much darker in color. Tissues presenting these characteristics should be discarded. They

probably would not grow at the same rate as tissues of homogeneous appearance, identical in area.

The condition of the edges of the fragments is also an important factor of the rate of growth. If the incision has been made through the growing area of the tissue, the periphery of the fragments has everywhere a similar appearance. The incision can be seen easily with the microscope if it has been made in places through the plasma not yet invaded by the new cells. Very often the old plasma is slightly folded. As the rate of growth is slower under these conditions such cultures should be discarded.

The value of the growth of a piece of tissue is compared only with the value of the growth of another piece of the same tissue. Therefore the absolute value of the growth is not of great importance, and the factors which do not modify the relative value of the growth can be neglected. However, these factors exist and may increase or decrease in a large measure the extent of the growth. In measurements made on 142 cultures, it was found that the surface of a fragment may increase from 4 to 40 times the initial size. These large differences in the amount of growth are due to the quality of the plasma and of the embryo extract used for the medium, to the previous condition of the strain, to the temperature of the incubator, and to many other factors. They are of no great interest, since they act at the same time on both the fragments which are to be compared. From a practical point of view, the factors which may influence independently the rate of growth of the two halves of an original fragment are almost completely eliminated, if the cultures containing pieces of old plasma or fragments of tissue of unequal size and thickness are discarded.

Present Condition of the Strain of Connective Tissue.

After more than 7 years of life outside the organism, the rate of growth of the strain of connective tissue is very active. This rate seems to have increased progressively during the 7 years (Figs. 1 and 2), but it is possible that it may be only an apparent increase due to modifications of the technique. During the 1st year the growth was slow and irregular, because it was not yet known that the presence in the culture medium of certain substances contained in embry-

onic juices is essential for the permanent life of tissues *in vitro*. As soon as the tissues were washed in Ringer's solution, or in salt solution every 2 days, and embryonic juice was used in the plasma of the medium, the rate of growth increased very much and became almost constant.² When the strain was 28 months old, the fragments of tissue which showed the maximum speed in growth increased in 48 hours 15 times their area. The 7 year strain increases more rapidly, as the area of a fragment may become in 48 hours 40 times larger than that of the original fragment. But this difference does not prove absolutely that the amount of new tissue produced in a given time is greater than it was 5 years ago. The medium used today is composed of one volume of plasma and one volume of embryonic juice, while 5 years ago it was composed of two volumes of plasma and one volume of embryonic juice. Since the medium is less dense, the growing tissue is probably thinner, and the increase in area does not mean the production of more tissue than that produced several years ago. The morphology of the cells has not varied. The photographs made 2 years ago and a few months ago show about the same number of mitotic figures (Figs. 3 to 5).

During the last 5 years the rate of growth fluctuated under the influence of the composition of the medium and the condition of the tissues. The plasma varied according to the age and the condition of the chicken and the embryonic juices were not of constant quality. Many other factors retarded or accelerated the growth of the tissue during periods of varying lengths of time. But as soon as the strain was again cultivated in normal medium, it grew at the normal rate. As has been shown previously, since the activity of the cells is a function of the medium in which they are living, it is readily modified by altering the composition of the medium. When the rate of multiplication of cells decreased and the amount of new tissue became small, the strain could be brought to normal growth within a few weeks. It was always found that the decrease in the rate of growth was due to deficient medium, or extract, or to the presence of alkali at the surface of the slides.

CONCLUSION.

1. A strain of connective tissue is still very active after more than 7 years of life *in vitro*.
2. The rate of growth of the fragments of tissue can be measured accurately and used for testing the action of many different factors on the growth of connective tissue cells.
3. The rate of growth of the strain is at least as rapid as it was 5 years ago, and may be more active.
4. The connective tissue cells appear to have the power of multiplying indefinitely in a culture medium, as do microorganisms.

EXPLANATION OF PLATES.

PLATE 51.

FIG. 1. Culture 9211-1. Passage 1080. Stained December 31, 1917. 48 hours growth. $\times 16$.

PLATE 52.

FIG. 2. Culture 13310-1. Passage 1367. Stained May 23, 1919. 48 hours growth. $\times 16$.

PLATE 53.

FIG. 3. Culture 5835. Passage 732. Stained April 16, 1917. 24 hours growth. $\times 275$.

PLATE 54.

FIG. 4. Culture 9211-1. Passage 1080. Stained December 31, 1917. 48 hours growth. $\times 275$.

PLATE 55.

FIG. 5. Culture 12784-1. Passage 1347. Stained April 25, 1919. 48 hours growth. $\times 240$.

108

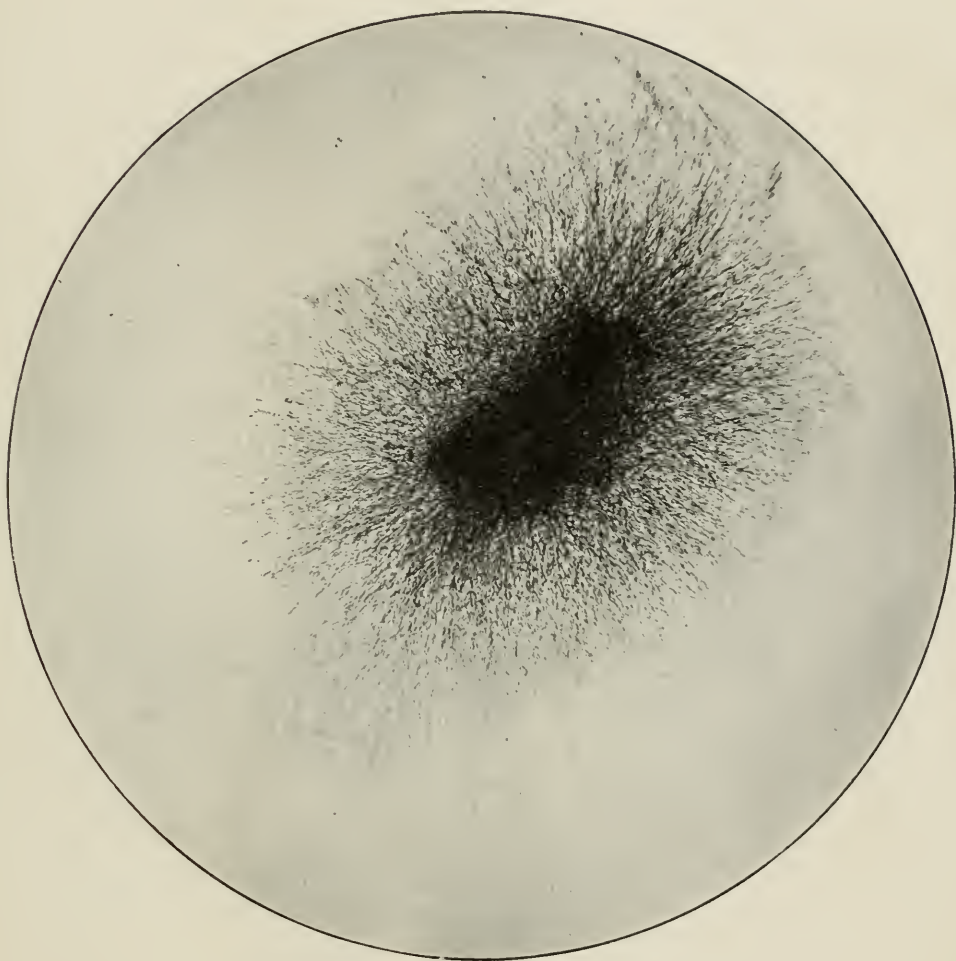


FIG. 1.

(Ebeling: Old strain of connective tissue.)

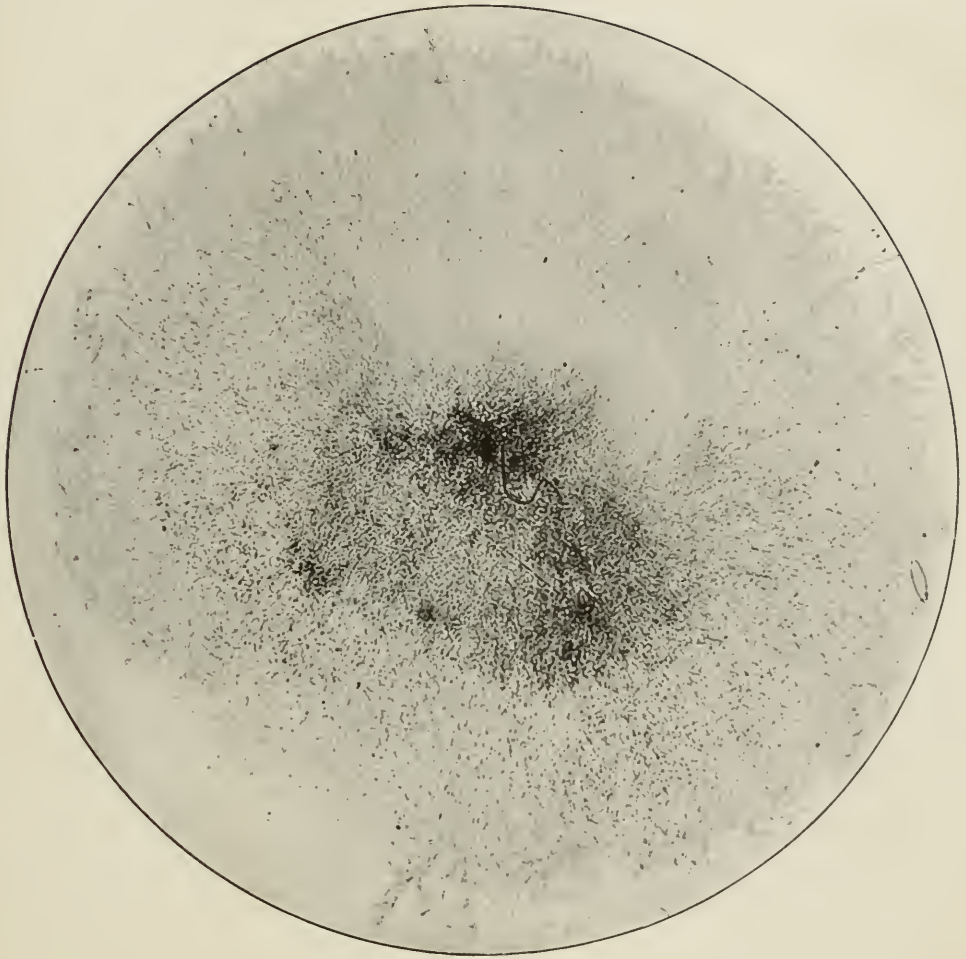


FIG. 2.

(Ebeling: Old strain of connective tissue.)

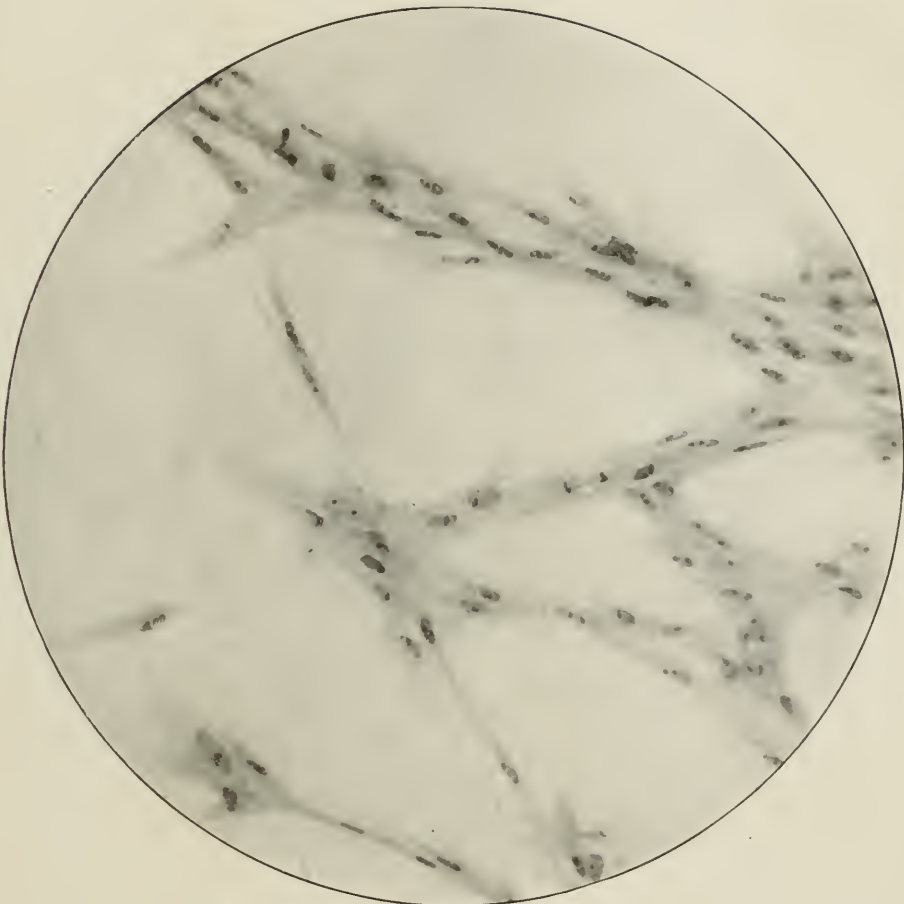


FIG. 3.

(Ebeling: Old strain of connective tissue.)

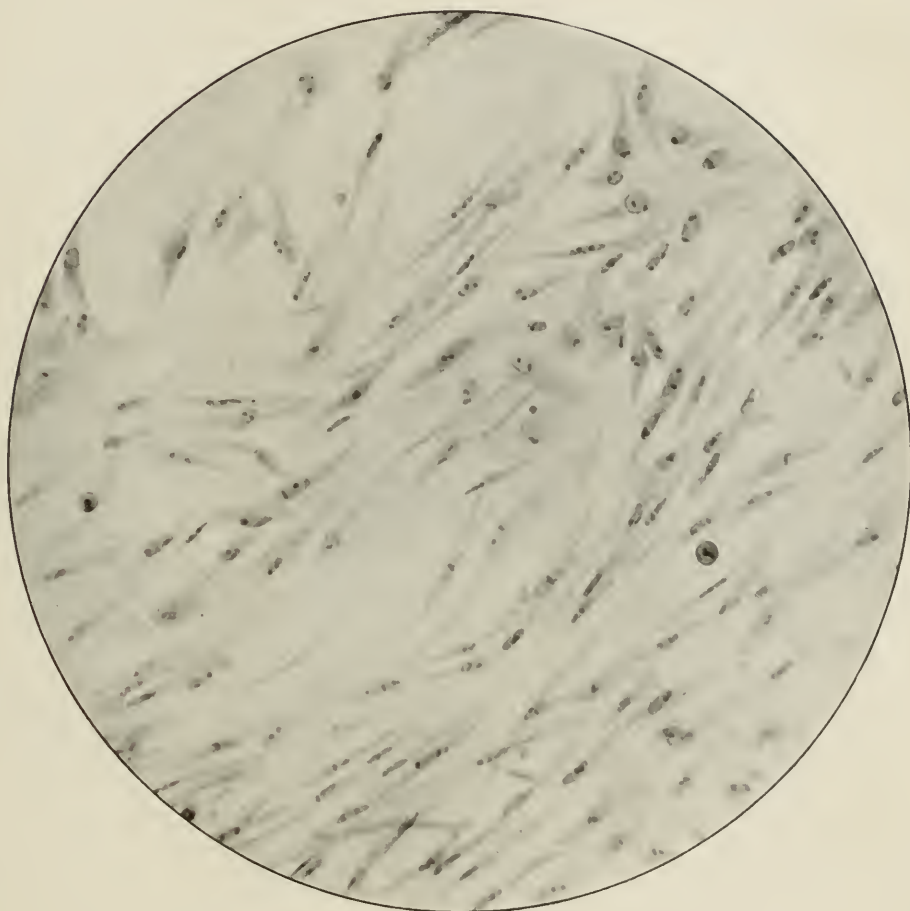


FIG. 4.

(Ebeling: Old strain of connective tissue.)

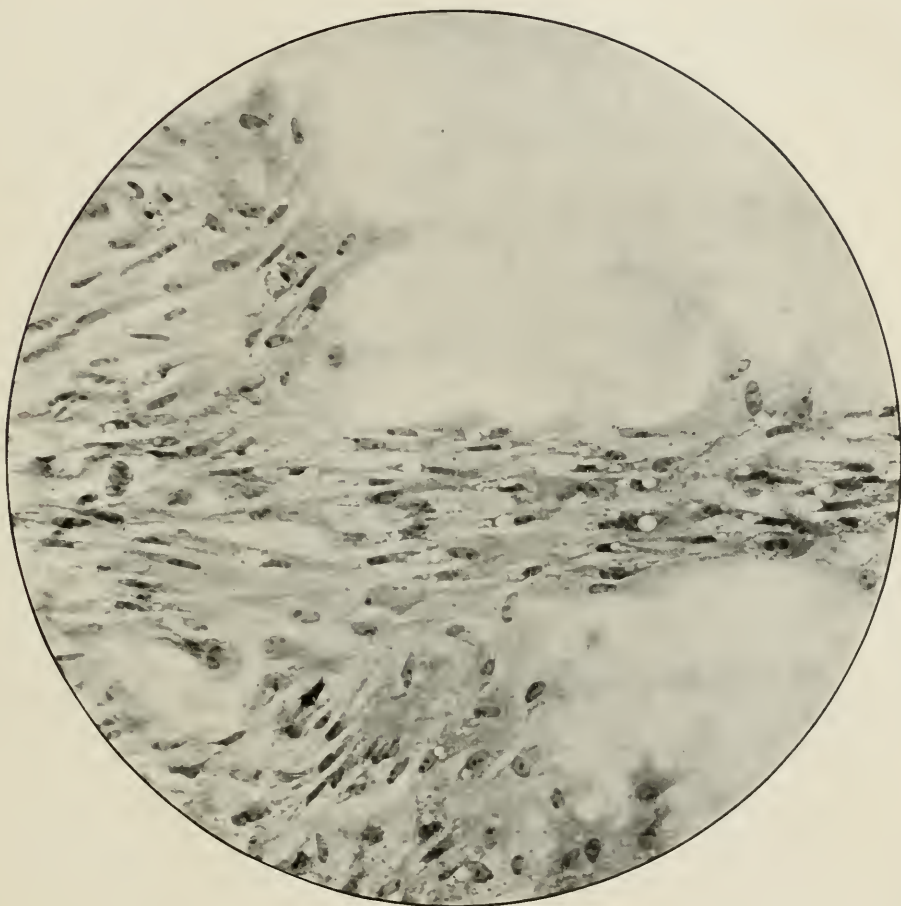


FIG. 5.

(Ebeling: Old strain of connective tissue.)

159

STREPTOLYSIN PRODUCTION IN CARBOHYDRATE MEDIA.*

BY F. A. STEVENS, M.D.,
Lieutenant, Medical Corps, U. S. Army,

AND S. A. KOSER, PH. D.,
Sergeant, Medical Corps, U. S. Army.

(From the Pathological Laboratory of Walter Reed General Hospital,
Takoma Park, D. C.)

(Received for publication, July 15, 1919.)

The inhibition of the hemolytic power of streptococci in media to which fermentable sugars have been added has been commented on by Ruediger (1), Sachs (2), Kuhn (3), Lyall (4), Davis (5), and Sekiguchi (6). After extended experiments Sachs concluded that in dextrose media the growth of the streptococcus was inhibited by the acid produced and the production of the hemolysin was correspondingly diminished. Kuhn, however, explained the effect of glucose by the theory that in carbohydrate media which were fermented by streptococci the metabolism of the organism changed so that the red cells were spared the direct lytic action of the bacteria. Although this in no way accounts for the diminished amount of hemolysin in filtrates of dextrose cultures, it was the first intimation that the protein-sparing action of carbohydrate might be applied to this phenomenon. Kendall and Farmer (7), studying the nitrogen metabolism, found that this principle was true in bacterial as well as animal physiology. Brown (8) has inferred that as there is undoubted evidence that streptolysin is a product of protein metabolism (it is thermolabile, can be filtered only through the coarsest filters, and is non-dialyzable), "in the presence of fermentable sugar the protein metabolism of the streptococci is reduced to a minimum and for this reason the formation of streptolysin is reduced or inhibited altogether." He suggested also that the acids formed might prevent the formation of streptolysin by acting on the growing streptococci, that the acids might inactivate the streptolysin during its formation, and, furthermore, might render the blood corpuscles insusceptible to hemolysis. It is possible on this basis to explain the action of sterile filtrates.

* From a report to the Surgeon General of the Army by a board of officers convened to study respiratory diseases.

In 1903 Schottmüller (9) noted that pneumococci and certain streptococci constantly produced green colonies on blood agar plates. Ruediger (1) attempted to show that this discoloration was due to acid production, and since the ferric chloride test on glucose broth cultures showed a reaction similar to that of lactic acid which he found would discolor blood media, he concluded that the green color was due to the action of lactic acid on red cells. He further states that not only did the addition of glucose increase the ability of pneumococci and streptococci to produce green colonies but that hemolytic cocci failed to hemolyze and after 36 to 48 hours incubation were surrounded by a distinct green halo. Cole (10) and Blake (11), working respectively with pneumococcus and *Streptococcus viridans*, discredited the action of acid in this reaction and showed that it was probably due to some inherent oxidative process acting on the cells immediately surrounding the colonies. Davis (5) found that after the addition of calcium carbonate to dextrose blood plates the average size of the zone of hemolysis was as large as on plain blood agar. By studying the solution of the carbonate particles and red cells simultaneously he concluded that the production of hemolysin and acid was coincident. Sekiguchi (6) stated that the hemolysin production was hindered by the glucose and not checked or destroyed by the acid. Finally, Brown (8) has observed that greenish or brownish discoloration similar to the alpha appearance is not produced by beta or gamma type of streptococcus in dextrose blood agar, but if there is sufficient fermentation the entire plate may be browned, first in the neighborhood of the colonies. The most noticeable effect was the inhibition of hemolysis by the actively hemolytic type. He further showed that acids produced in dextrose might account for some of the hemolysis of the gamma type streptococcus in poorly buffered media.

The reaction of the culture media for the production of streptolysin was described by Lyall (4) as optimum at 0.3 per cent acid to phenolphthalein, but he found inhibition if he varied the reaction much on either side of this point. M'Leod (12) obtained the greatest production in media distinctly alkaline to litmus. Since cultures become excessively acid in sugars which are fermented, calcium carbonate was added by Sachs (2) and Lyall (4), and the hemolysin determined. Lyall found that there was still marked inhibition. Sachs' tables show but little variation from the ordinary culture media except that the lysin was active slightly longer; by the addition of lactic acid to media before inoculation he shortened the life of the hemolysin by half. Braun (13) added acid and alkali to filtrates and after 6 hours incubation the alkaline preparation was most hemolytic; a filtrate obtained from a serum broth culture was made alkaline and incubated with a quantity of the original filtrate as a control. Both results were similar. Braun (13) and Sachs (2) concluded that the ability of the streptococcus to form acid was not to be identified with its hemolysin-producing power. That sufficient acid is formed in dextrose media to hemolyze red cells was shown by Sekiguchi (6); a 24 hour culture heated at 60°C. was not hemolytic after neutralization.

Methods.

The most important factor in the production of strong hemolysin is the media employed for the growth of the streptococcus. After testing various sera and broths M'Leod (12) considered beef infusion peptone broth with the addition of 20 per cent inactivated horse serum the most satisfactory, since he was able to obtain filtrates which were strongly hemolytic and fairly constant. In the following experiments a similar medium was used with 2 per cent peptone. No difficulty was experienced at any time in obtaining hemolysins and the filtrates were very active if obtained through new Mandler filters after 10 to 14 hours incubation. Since the object of the study was to observe the effect of dextrose and other fermentable sugars on the lysin production, it seemed necessary to measure the acid in the cultures at stated intervals. This was accomplished by means of a series of phosphate mixtures prepared according to Sørensen (14). To overcome the interference of the protein in the heavy serum media, 0.25 cc. was removed and diluted to 10 cc. before the indicators were added. The facts concerning the growth of the streptococcus were established on plate counts usually taken every 4 hours. It is realized that colony counts are open to much criticism, especially when an organism is counted which occurs in chain formation; films were made, a large number of groups of cocci was counted, and the number of individuals in each specimen was averaged. This factor usually varied from 4 to 7. In view of these facts, since the purpose was to obtain an idea of the relative growth of the organisms, the counts obtained by multiplying this number by the calculated colonies per cubic centimeter of the culture were sufficiently accurate. Hemolysis was determined by a series of tubes set up with increasing amounts of sterile filtrate or, as in the determination of hemolysin production, with the supernatant fluid obtained after centrifuging a portion of the culture at high speed for 10 minutes. 1 cc. of a 5 per cent suspension of washed human cells was added to each tube, and after an hour at 37°C. all were made up to 5 cc. volume and the undissolved cells were counted in a Levy chamber; the degree of red cell destruction was calculated in percentages by comparison with a standard control. Such counts are accurate since the occurrence

of shadow cells observed in hypotonic salt solution is rare; a perfect curve can be constructed from the degrees of hemolysis in the increasing amounts of hemolytic filtrate.

EXPERIMENTAL.

The effects of sugars were first observed on a series of plates made with 5 per cent defibrinated human blood and 1 per cent carbohydrate. The streptococci were obtained in pure culture from acute empyema fluids; five strains were chosen which gave a beta type hemolysis and had a final hydrogen ion concentration of pH 5.2 to 4.9 in dextrose broth. In liquid media they fermented dextrose, maltose, saccharose, lactose, and salicin, but did not so utilize mannite, inulin, glycerol, or raffinose. The first three sugars constantly inhibited hemolysis with discoloration of the media after 24 to 48 hours incubation, but lactose and salicin were more inconstant in action and some degree of cell destruction was observed at times without apparent cause. The typical green colony attributed to the pneumococcus or *Streptococcus viridans* was not observed. The remaining four substances had little effect on the size of the hemolyzed zone. Rarely the edges of the decolorized areas were not so sharp as on plain blood agar plates. It was apparent that the sugars which were easily fermented inhibited hemolysin formation most (Lyall (4)), but the resistant carbohydrates occasionally gave uncertain reactions. It is among this last group that the majority of sugars employed for the differentiation of various strains occurs; hence the appearance of the colonies on sugar blood agar plates does not seem accurate as a test of fermentation (Davis (5)). This inhibition of lysin production occurs in liquid as well as solid media. Sekiguchi (6) noted that distinct hemolysin was found only in plain broth, seldom in a weak content of glucose, and rarely in 3 per cent. The degree of inhibition is necessarily dependent to a large extent on the enrichment of the media with serum, since in simple media which are not suitable for the development of a strong hemolysin no trace is evident when an easily fermented sugar has been added previous to inoculation. In the following tests active hemolysin could always be obtained in the presence of carbohydrates.

Experiment 1.—Two flasks of media of 275 cc. volume, identical in every respect except that 1 per cent dextrose had been added to one, were inoculated with equal amounts of a 20 hour culture of Strain A. Bacterial counts, hemolysin determinations, and the hydrogen ion concentration were done at once and at intervals during a 48 hour period. The results are shown in Table I and further illustrated in Text-fig. 1.

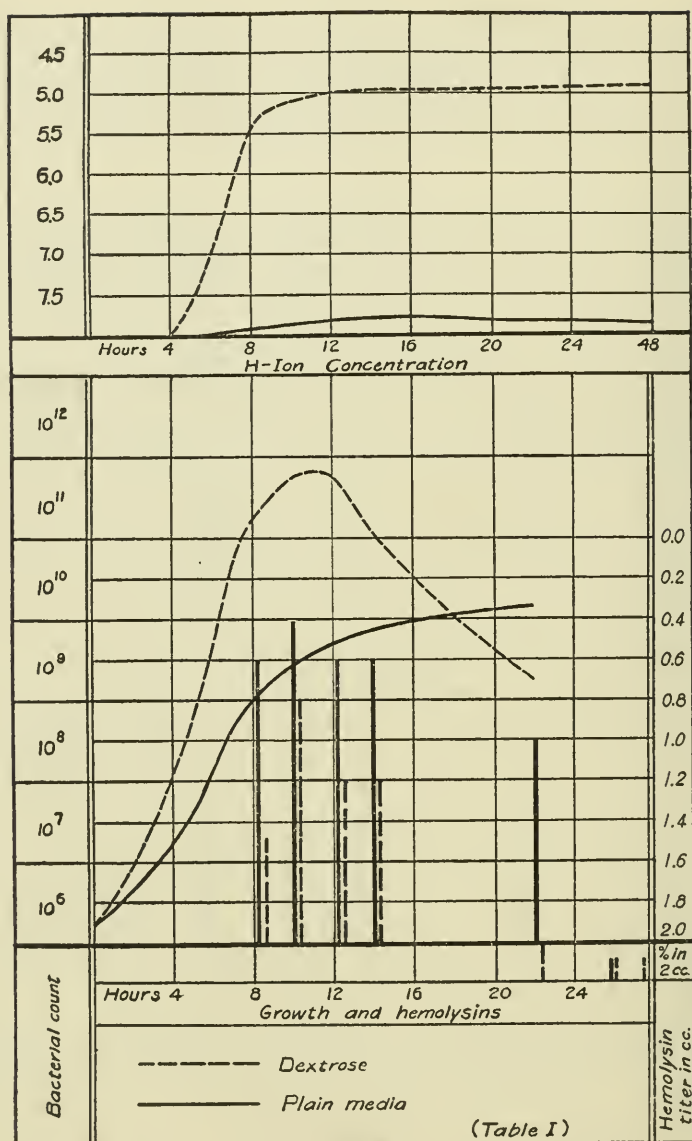
TABLE I.

Interval.	pH	Quantity of fluid.	Hemolysis.*	Bacterial count.†
Plain media.				
<i>hrs.</i>		<i>cc.</i>	<i>per cent</i>	
0	8.0	2.0	0	1.6(10 ⁶)
4	8.0	2.0	0	1.9(10 ⁷)
6	8.0	2.0	0	2.0(10 ⁸)
8	7.9	0.6	100	
10	7.9	0.4	100	
12	7.8	0.6	100	6.5(10 ⁹)
14	7.8	0.6	100	
22	7.8	1.0	100	1.1(10 ¹⁰)
28		2.0	50	
48	7.9	2.0	0	
Dextrose media.				
0	8.0	2.0	0	1.6(10 ⁶)
4	8.0	2.0	0	9.9(10 ⁷)
6	7.1	2.0	0	3.8(10 ⁹)
7	6.3			
8	5.5	1.5	100	2.1(10 ¹¹)
10	5.1	0.8	100	
12	5.0	1.2	100	7.8(10 ¹¹)
14	5.0	1.2	100	1.0(10 ¹¹)
22	4.9	2.0	100	2.6(10 ⁹)
28	4.9	2.0	50	
48	4.9	2.0	50	

* Hemolysins are indicated by the amount of supernatant fluid required to hemolyze 1 cc. of the red cell suspension or in the percentage of hemolysis if incomplete.

† The counts are given in powers of 10.

Experiment 2.—The previous experiment was repeated with three flasks, one of plain horse serum beef infusion media, one with 1 per cent dextrose, and one with dextrose and 1 per cent calcium carbonate. These were inoculated with



TEXT-FIG. 1. Comparison of the effects of plain media and dextrose media on lysin production.

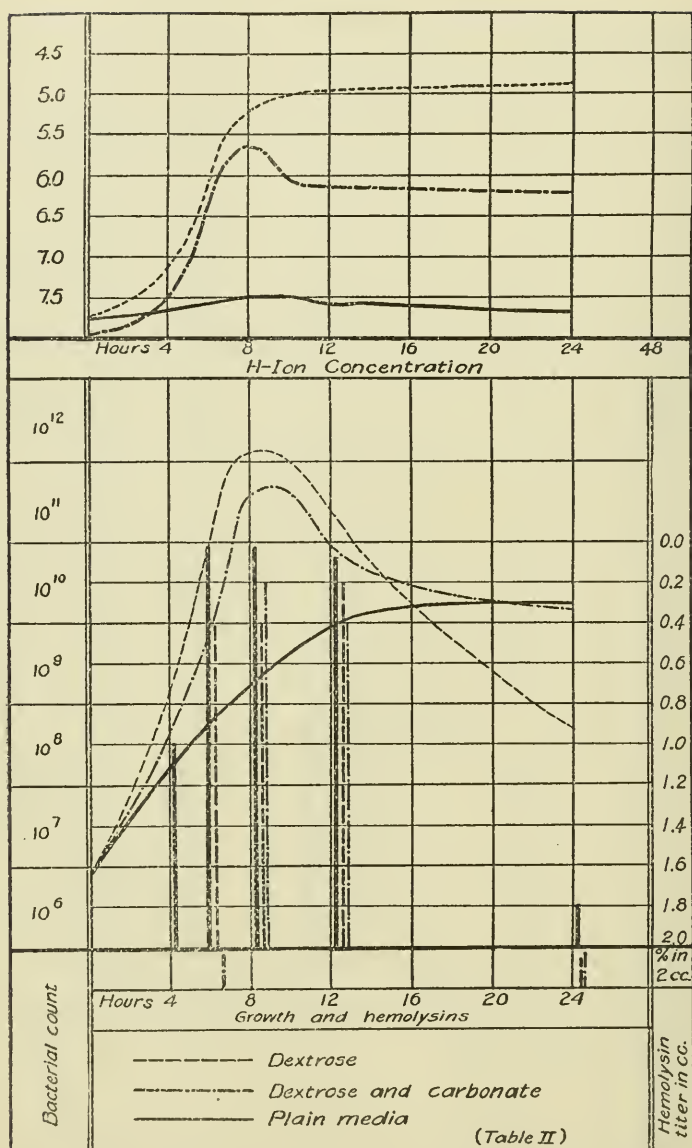
equal amounts of a 20 hour culture of Strain B. The results are illustrated in Table II and Text-fig. 2.

TABLE II.

Interval.	pH	Quantity of fluid.	Hemolysis.	Bacterial count.
Plain media.				
<i>hrs.</i>		<i>cc.</i>	<i>per cent</i>	
0	7.8	2.0	0	8.7(10 ⁶)
4	7.7	1.0	100	2.8(10 ⁸)
6	7.6	0.05	100	
8	7.5	0.05	100	2.9(10 ⁹)
10	7.5			
12	7.6	0.1	100	1.0(10 ¹⁰)
24	7.7	1.8	100	1.8(10 ¹⁰)
Dextrose media.				
0	7.8	2.0	0	8.7(10 ⁶)
4	7.3	2.0	0	1.3(10 ⁹)
6	6.1	0.4	100	
8	5.3	0.4	100	1.2(10 ¹²)
10	5.0			
12	5.0	0.2	100	4.1(10 ¹¹)
24	4.9	2.0	50	5.7(10 ⁸)
Dextrose carbonate media.				
0	8.0	2.0	0	8.7(10 ⁶)
4	7.5	2.0	0	5.7(10 ⁸)
6	6.3	2.0	75	1.5(10 ¹⁰)
8	5.6	0.2	100	7.0(10 ¹¹)
10	6.2			
12	6.2	0.4	100	6.2(10 ¹⁰)
24	6.1	2.0	75	1.0(10 ¹⁰)

Experiment 3.—Experiment 2 was repeated, beginning with media titrated to pH 7.6. The flasks were inoculated with equal quantities of a 20 hour broth culture of Strain B (Table III).

The growth of streptococci in dextrose media is much more rapid and profuse than in plain broth, and concomitant with this growth there is a rapid change in hydrogen ion concentration. In cultures which are not so heavily seeded as were those in Tables I to III the maximum acidity is reached within 24 hours (Avery and Cullen (15)).



TEXT-FIG. 2. Comparison of the effects of plain media, dextrose media, and dextrose carbonate media on lysin production.

TABLE III.

Interval.	pH	Quantity of fluid.	Hemolysis.	Bacterial count.
Plain media.				
<i>hrs.</i>		<i>cc.</i>	<i>per cent</i>	
0	7.6	2.0	0	9.2(10 ⁸)
3½	7.6			
4				3.7(10 ⁸)
5	7.5	1.2	100	
6	7.2	0.2	100	
8	7.3	0.1	100	3.6(10 ¹⁰)
11	7.3	0.05	100	
12				4.6(10 ¹⁰)
24	7.3	1.0	100	6.8(10 ⁹)
Dextrose media.				
0	7.6	2.0	0	9.2(10 ⁸)
3½	7.6			
4				2.4(10 ⁸)
5	6.6	2.0	0	
6	5.5	1.5	100	
8	5.0	0.4	100	5.0(10 ¹¹)
11	4.9	0.3	100	
12				1.9(10 ¹²)
24	4.9	2.0	75	3.2(10 ⁹)
Dextrose carbonate media.				
0	8.0	2.0	0	9.2(10 ⁸)
3½	8.0			
4				9.5(10 ⁸)
5	7.0	2.0	0	
6	6.1	2.0	50	
8	5.5	0.6	100	5.8(10 ¹³)
11	5.5	0.2	100	
12				2.7(10 ¹⁰)
24	6.4	2.0	50	5.1(10 ¹⁰)

The greatest increase occurs in the first few hours of incubation during the time that the hemolysin is produced. As the limiting acid concentration is approached the streptococci are found to be less viable, and from that time there is a gradual reduction in the count. Cultures in plain serum broth show a stronger hemolysin

titer during this period of active growth, while the production of hemolysin not only begins earlier but the cultures are actively hemolytic for a longer time. In general, the hemolysin curves obtained approximate the figures of Lyall (4), M'Leod (12), and Besredka (16). The addition of calcium carbonate allows a pH intermediate between those of flasks of plain serum bouillon and of flasks to which glucose alone has been added. With this partial neutralization the growth approaches or exceeds that in dextrose, but the production of hemolysin is still further delayed. In several experiments it was found that the rate of growth in the carbonate dextrose media was inverse to the hemolysin production, and, furthermore, in the cultures without sugar, although there were fewest streptococci, the hemolytic property was always greatest. It is apparent from Tables I to III that with the increased utilization of dextrose the hemolysin production is

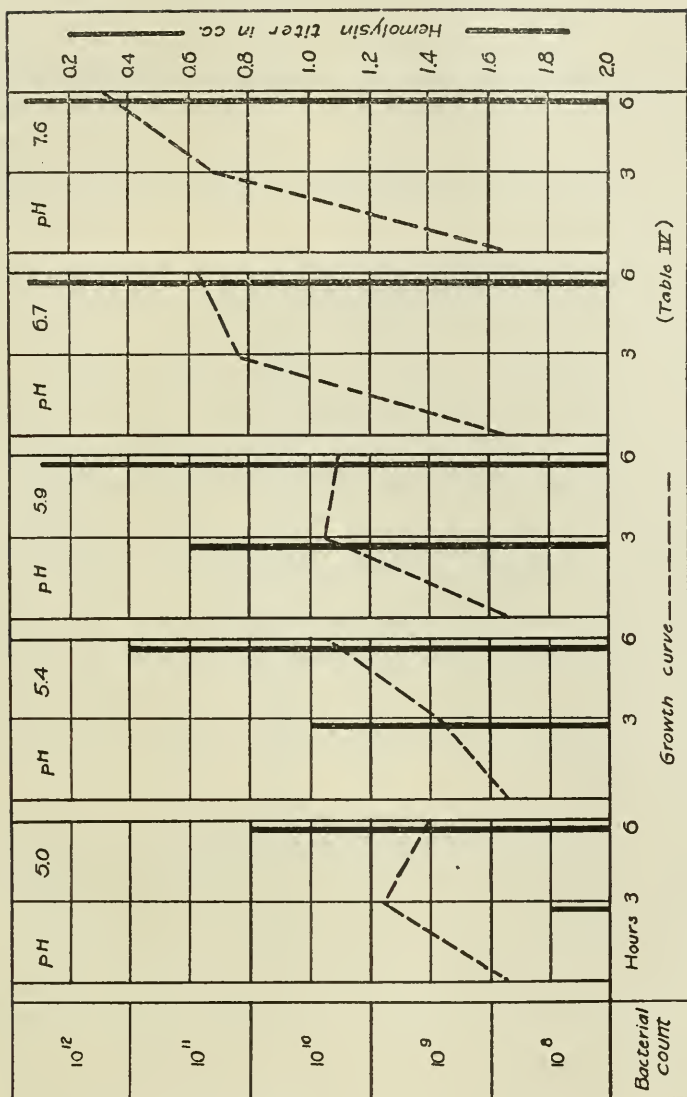
TABLE IV.

pH	3 hrs.		6 hrs.	
	Titer.	Bacterial count.	Titer.	Bacterial count.
	cc.		cc.	
7.6		2.6(10 ¹¹)	0.05	1.7(10 ¹²)
6.7		1.1(10 ¹¹)	0.05	3.8(10 ¹¹)
5.9	0.6	3.0(10 ¹⁰)	0.1	2.1(10 ¹⁰)
5.4	1.0	4.0(10 ⁹)	0.4	3.7(10 ¹⁰)
5.0	1.8	8.8(10 ⁹)	0.8	5.0(10 ⁹)

inhibited because of the protein-sparing action of the carbohydrate. The effects of the acids developed in the cultures still further explain these figures.

It was found that when the hydrogen ion concentration of the media was increased before inoculation by the addition of lactic acid, the growth of the streptococci and the hemolysin produced were proportional to the pH. This interferes in no way with the previous findings, since in the curves established the multiplication of the bacteria was not retarded in the first few hours of incubation, yet less lysin was produced. The effect of the acid in Table IV is primarily one of growth inhibition.

Experiment 4.—Five 100 cc. flasks of veal infusion horse serum media were used. The original titer of the media was pH 7.6. Four of the flasks were



TEXT-FIG. 3. The effect of acid on lysin production.

titrated to pH 6.7, 5.9, 5.4, and 5.0 respectively with 0.5 N sterile lactic acid. The volumes were then made equal with sterile 0.85 per cent sodium chloride solution. Each of the flasks was inoculated with the washed streptococci centrifuged from 20 cc. of an 18 hour culture of Strain B. Counts and hemolysin determinations were made after 3 and 6 hours incubation (Table IV, Text-fig. 3). The pH had not changed at the end of the period. Hemolytic titers are designated by the smallest amount of supernatant fluid which would completely dissolve 1 cc. of a 5 per cent suspension of human cells in 1 hour at 37°C. None of the flasks was hemolytic at the beginning. The counts before incubation were $8.5 (10^8)$.

The effects of acid are still more far reaching. Sterile hemolytic filtrates incubated in various strengths of acid for 6 hours lose a large part of their hemolytic property. That there is destruction of the hemolytic substance is evident, since after titration back to the original pH with sodium hydroxide the solution of the red cells is still less than that of the control tubes. The percentages of hemolysis in the tubes containing the lowest amounts of filtrate serve best as a means of checking this destructive action. In the determinations of hemolysis by the addition of 1 cc. of a 5 per cent suspension of blood cells in salt to small amounts of acid filtrate, the pH is raised to a point which in itself is not destructive to red cells. The acidity in the larger quantities is sufficient to account for the greater hemolysis in the tubes incubated at pH 5.0 for 6 hours. Supernatant fluids acidified and titrated back to neutrality with secondary sodium phosphate after incubation gave similar results. Braun's (13) tables also show this point.

Experiment 5.—An 18 hour culture of Strain B was found actively hemolytic. It was filtered through a new Mandler filter and divided into three equal portions. One was left at its original pH, 7.4. The others were adjusted to a pH of 5.0 and 6.0 by the addition of sterile 25 per cent lactic acid. The hemolytic titer was determined at once and after 3 and 6 hours incubation (Table V). At the end of 6 hours the flasks were neutralized (7.4) and the volumes were made up by the addition of normal salt solution so as to contain an equal concentration of the original filtrate. Cultures of the flasks at the beginning and at the end of the experiment were sterile.

In Table V it was assumed that some of the hemolysis in the strongly acid tubes was due to acid concentration. It was observed that salt solution, to which acid had been added until a pH was reached which

would check the growth of streptococci, had no effect on red cells. If red cells were incubated 1 hour in media made to contain a large quantity of weakly ionized electrolytes and titrated the same way with lactic acid, the solution and discoloration were striking. The destruction of corpuscles begins at about pH 6 and in broth is complete

TABLE V.

Quantity of filtrate.	Hemolysis.		
	pH 5.0	pH 6.0	pH 7.4
Hemolysis before incubation.			
<i>cc.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.05	100	100	100
Hemolysis after 3 hrs. incubation.			
0.8	100	100	100
0.4	98	100	100
0.2	96	100	100
0.1	78	98	99
0.05	54	96	98
Hemolysis after 6 hrs. incubation.			
2.0	99	87	99
1.0	98	87	98
0.6	84	84	98
0.2	50	68	92
0.1	40	59	86
0.05	30	43	85
Titrated back to pH 7.4 after 6 hrs.			
1.5	28	11	99
1.0	0	22	98
0.8	0	7	97
0.4	0	1	94
0.2	0	0	92

at pH 5. Coincident with this acid hemolysis the hemoglobin is turned brown. Due to the fact that there is so much cell destruction it is impossible to determine the effect of hemolysin on blood cell suspensions which have been previously treated with acid. Lyall (4) has already noted that cells which have been discolored by actively

TABLE VI.

	Control.	0.5 per cent.	1 per cent.	2 per cent.
Filtrate	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.
Sterile sugar solution (10 per cent)	0.0 "	0.05 "	0.1 "	0.2 "
Saline solution	0.9 "	0.85 "	0.8 "	0.7 "

Hemolysis before incubation.				
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Dextrose	99	99	98	98
Mannite	100	97	97	96
Invert sugar	100	99	99	99

Hemolysis after 3 hrs. incubation.				
Dextrose	97	92	81	82
Mannite	96	95	90	82
Invert sugar	98	96	98	94

Hemolysis after 6 hrs. incubation.				
Dextrose	95	84	61	64
Mannite	87	76	70	61
Invert sugar	90	88	88	83

<i>Series with Large Quantities.*</i>				
Quantity of filtrate.	Control tube.	Dextrose.	Mannite.	Invert sugar.

Hemolysis after 3 hrs. incubation.				
<i>cc.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.8	100	100	100	100
0.6	99	100	99	99
0.2	99	100	97	98
0.05	97	97	95	96

Hemolysis after 8 hrs. incubation.				
2.0	95	96	95	97
1.0	90	93	90	95
0.6	85	87	86	89

* Cultures were sterile after incubation.

growing pneumococci are more resistant to streptolysin, but this action of the pneumococcus, mentioned by Cole (10), probably enters very little into the discoloration of hemoglobin in dextrose cultures of the beta type streptococcus.

The presence of either dextrose, mannite, or invert sugar exerted a slight inhibition on hemolytic filtrates. This cannot account for any of the marked differences in the titer of broth cultures or in the gross appearance of blood agar plates, but it is probably the cause of the hazy edges occasionally observed in the hemolyzed zones on solid media containing unfermented sugars. It is impossible to demonstrate this effect on large quantities of filtrate, but if the minimal hemolytic amount is incubated in 1 per cent of these sugars the solution of the red cells is much retarded after a few hours (Table VI).

Experiment 6.—The effect of sugars on hemolytic filtrates was studied in the following way. It was found that 0.05 cc. of a sterile filtrate obtained from a 12 hour culture of Strain C caused complete hemolysis of 1 cc. of the red cell suspension. A series of tubes was set up containing 0.1 cc. of the filtrate in 1 per cent dextrose, mannite, and invert sugar; the tubes were incubated 3 and 6 hours. The hemolysin tests are indicated in Table VI. To determine the effect of these substances on larger quantities of filtrate, 50 cc. lots were treated in the same manner and the titer was determined after 3 and 8 hours.

SUMMARY.

It is evident that there are numerous interacting factors which affect streptolysin in the presence of fermented sugars. The principal action is the change in the metabolism of the streptococcus by which more carbohydrate and less protein is utilized; although the growth is much increased there is proportionately less hemolysin. The acid developed in these cultures not only lessens the vitality of the growing organism and so lessens proteolysis, but is destructive to hemolysin at incubator temperature. The concentration of the acid produced causes some hemolysis, and a coincident brown discoloration of the hemoglobin. These principles may be applied to both liquid and solid media.

BIBLIOGRAPHY.

1. Ruediger, G. F., *J. Infect. Dis.*, 1906, iii, 663.
2. Sachs, E., *Z. Hyg. u. Infektionskrankh.*, 1909, lxiii, 463.
3. Kuhn, F., *Centr. Bakteriolog., 1te Abt., Orig.*, 1912, lxiii, 97.
4. Lyall, H. W., *J. Med. Research*, 1914, xxx, 515.
5. Davis, D. J., *J. Infect. Dis.*, 1917, xxi, 308.
6. Sekiguchi, S., *J. Infect. Dis.*, 1917, xxi, 475.

7. Kendall, A. I., and Farmer, C. J., *J. Biol. Chem.*, 1912, xii, 13, 215.
8. Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, 1919.
9. Schottmüller, H., *Münch. med. Woch.*, 1903, l, 849.
10. Cole, R., *J. Exp. Med.*, 1914, xx, 363.
11. Blake, F. G., *J. Exp. Med.*, 1916, xxiv, 315.
12. M'Leod, J. W., *J. Path. and Bacteriol.*, 1911-12, xvi, 321.
13. Braun, H., *Centr. Bakteriolog., 1te Abt., Orig.*, 1912, lxii, 383.
14. Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.
15. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.
16. Besredka, *Ann. Inst. Pasteur*, 1901, xv, 880.

PFEIFFER'S BACILLUS AND INFLUENZA.

A SEROLOGICAL STUDY.

BY MARTHA WOLLSTEIN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 15, 1919.)

The literature which has grown up in the very short time since the pandemic of influenza in 1918 is already so voluminous that it cannot be abstracted and analyzed in a brief space. Moreover, such an analysis, at the present time, would not be profitable. The methods employed by different bacteriologists in cultivating the influenza bacillus of Pfeiffer have been so diverse that comparison of results is valueless. There is, at present, hardly any difference of opinion that, with an adequate bacteriological technique, Pfeiffer's bacillus is found to be very commonly present in the respiratory tract of persons suffering from influenza and its attendant pneumonia. Moreover, the bacillus is also widespread in the upper respiratory mucous membranes of persons who have not had influenza, and even of persons who have not, so far as is known, been exposed to the epidemic disease.

We may accept the wide prevalence of the so called influenza bacillus as established and admitted and then proceed to the next and more important, because essential, question of the relationship of the bacillus to the symptom-complex influenza. Except in epidemic periods this symptom-complex is not so definite as to enable a sure and prompt diagnosis of influenza to be made; hence the cultivation of the Pfeiffer bacillus in interepidemic periods, from the upper respiratory tract, is not significant, necessarily, of clinical influenza. Possibly, indeed, there is no pathogenic microorganism, except the omnipresent pyogenic cocci, so frequently present in those parts as the Pfeiffer bacillus.

But even though the etiological rôle of the Pfeiffer bacillus in influenza has come to be seriously questioned, the part it plays in the

pathological complex characterizing this severe disease may still call for definition. Fortunately, in respect to this point there are other means available than mere presence in cultures to determine pathogenic action. The study on which this paper is based was begun early in the epidemic. In the meantime other publications bearing on the same topic have appeared. But it would seem that the subject is one on which additional evidence and more varied tests are desirable; hence the presentation of our results.

Culture Media.

Rabbit blood agar was the routine medium used in searching for Pfeiffer's bacillus during life and at autopsy, until Avery's oleate agar¹ became available. For serological work a medium made in the following way proved most suitable. Fresh rabbit blood was boiled for 2 minutes in a water bath, then centrifuged. The resulting clear, pale pink or yellow fluid was found by spectroscopic examination to contain hemoglobin. Two or three drops were added to 5 cc. of melted agar (pH 7.5) and slanted, while 0.5 cc. was added to a tube containing 20 cc. of broth (pH 7.8). Thus a solid medium and a fluid medium were obtained in which Pfeiffer's bacilli grew abundantly, and washing to get rid of blood or serum was not necessary. The bacilli live only 5 to 6 days on this medium, however. To keep stock cultures, blood broth, in which the bacilli remain viable for 6 weeks or more, proved by far the most suitable medium, while for isolation of the organisms the oleate agar was best.

Serological Reactions.

It was clear that for serological study sera from convalescent patients as well as monovalent immune sera experimentally produced were needed. Rabbits weighing 1,600 gm. proved to be best suited for the purpose of immunization. By means of intravenous injections of live Pfeiffer bacilli, in increasing doses, administered every 3rd day, antisera were produced with ten strains, seven isolated during the epidemic and three obtained several years ago.

¹ Avery, O. T. *J. Am. Med. Assn.*, 1918, lxxi, 2050.

Agglutination.—It was difficult to get even suspensions of Pfeiffer's bacillus suitable for agglutination tests. Blood broth cultures were not sufficiently profuse, and cultures grown on solid medium clumped spontaneously whether washed or not. Centrifuging had the effect of making the bacilli stick together in small clumps which could not be broken up. Finally, bacilli grown on the boiled-blood-centrifuge-agar were suspended in distilled water, while the serum dilutions were made in normal salt solution. The mixtures were kept at 55°C. for 2 hours, then at room temperature over night. At the end of 2 hours no agglutination had taken place; consequently the 18 to 20 hour reading was the only one that could be noted. The controls obtained by this method were fair for most strains, but unsatisfactory for others. Three strains clumped so much spontaneously that they could not be used for agglutination tests. The strains of Pfeiffer's bacillus varied in the degree of their agglutination reactions, as well as in the kind; four were inagglutinable in every serum tested, one agglutinated only in its homologous monovalent rabbit serum in dilutions of 1:100, while three other strains clumped in 1:50 dilutions in that serum. The presence of higher agglutinin content for heterologous than for homologous strains was noted for three sera made with strains of Pfeiffer's bacilli isolated from epidemic cases.

One normal rabbit serum was encountered which agglutinated the Pfeiffer bacillus in a dilution of 1:20.

The serum of five normal human adults did not agglutinate Pfeiffer's bacillus in any dilution, while two other normal sera reacted in dilutions not exceeding 1:10. Sera from eleven patients were tested during the 2nd week of illness. Five had had a mild bronchopneumonia attending the influenza attack, and the serum from two of these agglutinated Pfeiffer's bacilli in dilutions of 1:100, while the other three sera did not contain agglutinins for these bacilli in dilutions higher than 1:40. Sera from six cases of simple influenza without pneumonia contained no agglutinins for Pfeiffer's bacillus in two instances, and reacted in dilutions of 1:40 in four others.

Agglutination reactions with the bacillus of Pfeiffer were not satisfactory because of the tendency to spontaneous clumping of the organisms. The wide variations in the results recorded in the litera-

ture may depend partly on this difficulty. Fleming² studied twenty-one influenza patients and found the agglutinin content of their sera low; only six agglutinated Pfeiffer's bacillus in dilutions higher than 1:32, and of these only one reacted above 1:128, giving a positive reaction in a dilution of 1:1,000. He also found that strains differed in their ability to agglutinate.

Adults who had been inoculated with vaccine made from several strains of Pfeiffer's bacilli developed agglutinins in their blood within 6 to 10 days, but only rarely were these present in dilutions higher than 1:100.

Complement Deviation.—Antigens were made in two ways: (a) Cultures grown in blood broth were centrifuged to throw out the blood cells, and the supernatant fluid was pipetted off, heated to 55°C. for 45 minutes, and tested. Against this antigen controls with sterile broth were always done in parallel series.

(b) Cultures in boiled-blood-coagulate-broth were centrifuged for 30 minutes and the precipitate was washed twice in salt solution, then resuspended in fresh salt solution and tested. The use of tricresol was discarded because it tended to make the antigens anticomplementary. A standard of turbidity for the suspended washed bacilli was evolved which approximated 10 cc. of salt solution to the precipitate from 25 cc. of broth culture. However, this amount had to be adjusted to the individual growth.

Tests with cultures of varying ages showed that the maximum strength of antigen was obtained in 3 days. The strains of Pfeiffer's bacilli varied in their antigenic power. In seven instances the antigen was strong, in eight, weak; while seven other strains failed to yield an active antigenic product. Whenever possible, sera were tested against antigen made from (a) a meningeal strain isolated during the epidemic, and (b) another meningeal strain isolated 2 years ago; (c) three respiratory epidemic strains; (d) a sporadic respiratory strain; (e) a strain isolated from a healthy carrier. Blood from forty-four persons was tested.³ Of these, fourteen were patients convalescent 6 to 23 days after the onset of an attack of influenza, nineteen

² Fleming, A., *Lancet*, 1919, i, 138.

³ I am indebted to Dr. Walter W. Palmer for his courtesy in allowing me to obtain blood from several patients in the wards of the Presbyterian Hospital.

had recovered from influenza 1 to 4 months previously, four were healthy carriers who had never been ill with influenza, and four were healthy controls. One fatal case of influenzal meningitis was studied, and also two patients who had suffered from diseases other than influenza—lobar pneumonia in one instance, laryngitis and pharyngitis in the other.

Sera from four normal adults were tested as controls. They had not been ill during the past fall and winter, had not suffered from colds, had not been vaccinated against influenza, and carried no Pfeiffer's bacilli in their sputum. No complement-binding reaction was obtained with any antigen in any of these four sera.

Sera from convalescent patients were examined at the end of the 1st week and reexamined in three cases at the end of the 2nd week after onset. Dilutions of 1:5, 1:10, and 1:20 were used and showed a diminishing strength of reaction. The results are shown in Table I. All the sera gave a fixation reaction on the 6th or 7th day, and all but two reacted with antigens made from more than one strain of Pfeiffer's bacillus. The three sera which were obtainable a second time reacted more strongly at the end of the 2nd week than on the 6th day. Five were mild cases without any signs of bronchopneumonia, while seven were complicated by pneumonia. It is to be noted that the sera of patients who suffered from pneumonia complicating influenza had stronger complement-binding power than did the sera of uncomplicated cases. No convalescent case failed to show fixation antibodies at the end of a week, though these were sometimes small in amount and limited in kind. No differentiation of strains was possible from these results.

One patient had been inoculated 5 weeks before she became ill with influenza with a vaccine of Pfeiffer's bacillus only. Her serum gave a reaction with but one antigen of five used. The influenzal attack had been a mild one, and apparently the inoculation did not increase the complement-binding power of her serum in the presence of Pfeiffer bacillus antigen.

An infant with Pfeiffer bacillus (influenzal) meningitis, who died on the 7th day of illness, gave a strongly positive reaction on the 6th day, with two antigens, one made from a meningeal strain of Pfeiffer's bacillus isolated 2 years before, and the other made from a strain

TABLE I.

Complement Fixation with Blood of Convalescent Patients.

Individual No.	Vaccine.	Day of illness.	Serum dilution.	Antigens.							
				M	D	325	R	W	T	I	280
1	1 mo. before.	9th	1: 5	0	+++	0		0			0
			1: 10	0	+++	0		0			0
			1: 20	0	+	0		0			0
2	0	9th	1: 5	+++	+++	+++		+++			0
			1: 10	+++	+++	++		++			0
			1: 20	+	++	+		0			0
3	0	7th	1: 5	0	+	++	++	++	++	++	++
			1: 10	0	+	++	+	+	++	+	0
			1: 20	0	0	+	0	0	0	0	0
	0	13th	1: 5	+	+++	+++	+++	+++	+++	+++	++
			1: 10	0	+++	+++	+	++	++	+	0
			1: 20	0	++	++	+	+	0	0	0
4	0	6th	1: 5	0	+	0	0	0		0	0
			1: 10	0	+	0	0	0		0	0
			1: 20	0	0	0	0	0		0	0
	0	12th	1: 5	0	+++	0	0	0		0	0
			1: 10	0	+++	0	0	0		0	0
			1: 20	0	++	0	0	0		0	0
5	0	6th	1: 5	+	++	0		0		0	0
			1: 10	0	+	0		0		0	0
			1: 20	0	0	0		0		0	0

W is a strain of Pfeiffer's bacillus isolated from a case of influenzal meningitis during the epidemic; T a strain isolated from a case of influenzal meningitis 2 years ago; M and D strains isolated from the lungs of influenzal pneumonia cases during the epidemic; 325 and R strains isolated from the sputum of influenzal pneumonia cases during the epidemic; I a strain isolated from the lungs of an influenzal pneumonia case 3 years ago; and 280 a strain isolated from the throat of a healthy adult carrier.

0 indicates complete hemolysis, no fixation of complement; + + + no hemolysis, complete fixation of complement; + + and + varying degrees of fixation of complement.

TABLE I—*Concluded.*

Individual No.	Vaccine.	Day of illness.	Serum dilution.	Antigens.							
				M	D	325	R	W	T	I	280
5	0	12th	1: 5	++	++++	0		0		0	0
			1: 10	0	++++	0		0		0	0
			1: 20	0	+	0		0		0	0
6	0	7th	1: 5	0	++++	++	++++	++++			
			1: 10	0	++++	++	++++	+			
			1: 20	0	++	0	+	+			
7	0	8th	1: 5	0	++++	++++	++++	++++			
			1: 10	0	++++	++	++++	+			
			1: 20	0	++++	0	+	0			
8	0	14th	1: 5	0	++++	++++	0	++++			
			1: 10	0	++++	+	0	+			
			1: 20	0	+	0	0	+			
9	0	6th	1: 5			++++			++		
			1: 10			++++			++		
			1: 20			+			+		
10	0	9th	1: 5	+	++++	++	++	++++	++++	++	++
			1: 10	0	++++	++	+	++++	+	+	+
			1: 20	0	+	0	0	++	0	0	0
11	0	7th	1: 5		++++					+	
			1: 10		+					+	
			1: 20		0					0	
12	0	14th	1: 5	++	++++				++	++	
			1: 10	++	++++				+	+	
			1: 20	0	++++				0	+	
13	0	13th	1: 5		++++					++	
			1: 10		++++					+	
			1: 20		++					+	
14	0	23rd	1: 5	++++	++++	++++	++		++	++	++
			1: 10	++	++++	++++	+		++	++	+
			1: 20	+	+	++	+		++	++	+

recovered from the lung in a fatal case of bronchopneumonia occurring during the epidemic. With antigen of a strain of Pfeiffer's bacillus from a healthy carrier, isolated during the course of the epidemic, and with that of a respiratory strain isolated from a sporadic case 4 years ago, no reactions were obtained. It is to be regretted that it was not possible to obtain a larger quantity of serum from this baby, so that it might have been tested with antigen made from its homologous strain of Pfeiffer's bacillus. The results obtained with this serum confirm the point previously shown;⁴ namely, that meningeal strains of Pfeiffer's bacillus are virulent and yield a strong antigen, and that while respiratory strains are more apt to be non-virulent and yield a weak antigen, virulent respiratory strains may be encountered which yield a strong antigen.

Complement fixation tests with Pfeiffer bacillus antigen made with the blood of nineteen individuals who had entirely recovered from influenza, which had attacked them 1 to 4 months previously, gave irregular results. At the end of a month one serum gave only an incomplete reaction with two antigens, and after 6 and 7 weeks only low complement-binding content was found in two other sera. 2 months after the illness no reaction was obtained with one serum, while three others gave strong reactions even in dilutions of 1:20, and a fourth reacted well in a dilution of 1:5, but not higher. After 3 months one serum gave complete reactions only in dilutions of 1:5, while another was positive in dilutions of 1:10. 4 months after influenza two sera were entirely devoid of complement-binding body content.

1 month after the influenzal illness a man who had been inoculated with three doses of vaccine made with three strains of Pfeiffer's bacillus, 4 months before the attack began, gave very strong reactions in dilutions of 1:10. 2 months after the attack of influenza the sera of two subjects inoculated with Pfeiffer bacillus vaccine 3 months before the illness reacted as strongly as did two unvaccinated patients, while one reacted much less well. In comparing the serum of inoculated and of non-inoculated individuals, it becomes evident that the administration of a vaccine, made from several strains of Pfeiffer's

⁴ Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.

bacillus, did not have any apparent influence on the degree of the complement-binding reaction with the antigens of Pfeiffer's bacillus when an interval of 3 or 4 months had elapsed between the inoculation and the influenzal illness. The patient whose serum was poor in this fixing antibody 1 month after the attack is apparently the exception, when viewed in the light of Table I.

Sera from four carriers were tested. All denied any respiratory illness in the previous 6 months. One had been inoculated with vaccine made from Pfeiffer's bacilli only. Two of the four carriers gave no reaction with several antigens. The inoculated individual and one person who had not been vaccinated reacted strongly. One of the carriers whose serum was negative to this test received Pfeiffer bacillus vaccine, and 1 week after the third dose had been injected his serum contained complement-binding bodies for three of six antigens against which it was tested.

To sum up, complement-binding antibodies were absent from the blood of four normal individuals who had not had influenza, and who were not carriers of Pfeiffer's bacillus. In the blood of influenza patients the fixing antibodies were present at the end of the 1st week, increasing in strength during the 2nd week, and were, as a rule, demonstrable at the end of 3 or 4 weeks. At the end of the 2nd month after an attack of influenza these antibodies were strongly present except in one case. At the end of the 3rd month complement fixation was still demonstrable in patients' serum, but in 4 months it had entirely disappeared. A complicating pneumonia increased the complement-binding power of the serum.

Monovalent immune rabbit sera were made with ten strains of Pfeiffer's bacillus and all bound complement in the presence of antigens made both from homologous and from heterologous strains. Normal rabbits were used as controls and their sera gave negative results.

Precipitins.—With the same antigen as that used for the complement fixation test, precipitins were demonstrable in every convalescent patient's serum tested, while they were absent from the serum of normal persons. Antigens made from homologous strains of Pfeiffer's bacillus gave no stronger precipitin reactions than did those made from heterologous strains. The earliest precipitin reaction noted was on the 6th day of the illness, and the latest, 3 months after the attack.

Poisonous Filtrates.

According to Parker⁵ filtrates of Pfeiffer bacillus cultures grown in heated blood broth for 6 to 20 hours are poisonous for rabbits when inoculated intravenously. Tests were made with twenty-five strains of the bacilli. The filtrates from seven strains killed rabbits weighing 1,300 to 1,600 gm. within 1 to 2½ hours when inoculated intravenously in doses of 2 or 3 cc. Much larger doses (5 cc.) were required to kill the animals with filtrates of other strains. Death was sometimes, though rarely, delayed until 12 to 15 hours after the injection of the poison.

The effects of intravenous injection of a potent filtrate from an early culture of Pfeiffer's bacillus were quite uniform. The animals gradually became more and more quiet, so that in 30 to 45 minutes they sat in a hunched up position with no movement but that of very rapid respiration. Intestinal peristalsis was increased until at the end of 60 to 90 minutes the animals were passing fluid feces. Gradually the head leaned, then fell to one side, and the animal slowly fell over. After one or two attempts to rise, all muscular efforts ceased, and it lay on its side, breathing very rapidly and irregularly, with the head becoming more and more retracted. Death was often preceded by general convulsions. Non-lethal doses caused the symptoms of muscular weakness, irregular respiration, retraction of the head, and diarrhea; but the rabbit did not fall over on its side or go into a convulsion, and recovery gradually took place so that the animal was well within 3 or 4 hours, though more quiet than before the injection. The leucocytes fell within 1 hour after inoculation, and a difference of 5,000 to 12,000 from the initial count was noted. The recovered animals showed a rise within 4 to 5 hours, and on the following day the leucocytes were always as high or higher than before the injection of the filtrate.

Postmortem examination showed constant changes in the lungs. These organs were deep pink in color, mottled with dark red or brown areas of hemorrhage, often most marked in one or both lower lobes. Punctate subpleural hemorrhages were numerous and general. The lungs were edematous; frothy fluid exuded from the cut surface and

⁵ Parker, J. T., *J. Am. Med. Assn.*, 1919, lxxii, 476.

from the trachea and bronchi. The mucosa lining the larynx, trachea, and large bronchi was dark red or purple in color from an intense congestion, while bright red points of capillary hemorrhages were numerous.

The intestinal contents were fluid. The kidneys and liver were congested. In female rabbits the Fallopian tubes were always bluish red in color and on section showed an intense congestion of all the coats. The blood vessels in the cerebrospinal meninges were filled with blood. No gross hemorrhages were apparent in the nervous system. The suprarenals were pale pink in color.

Microscopic examination of the brain showed minute areas of perivascular diapedesis in sections of the medulla. The lungs showed a congestion of all the vessels so exquisite as to constitute a natural injection. A comparative absence of leucocytes was striking; both within the lumen of the vessels and in the alveoli they were few in number. The connective tissue septa were edematous. Many alveoli contained red blood cells, and the epithelium lining the walls was intact; other alveoli contained granular, coagulated serum in which air bubbles were apparent.

In the female the Fallopian tubes showed an intense congestion of the blood vessels in the mucosa, while some muscle fibers had undergone hyaline degeneration.

Control experiments were made by injecting normal incubated broth intravenously into rabbits. No symptoms followed, and at autopsy no lesions were found. A filtrate of typhoid bacillus culture was also injected as control. The animals died after 15 to 18 hours, and at autopsy showed a hypostatic congestion of the lungs and some discoloration due in large part to the fact that they had been dead several hours before they were examined. The edema and punctate hemorrhages were lacking.

The sera of rabbits inoculated with increasing doses of filtrates of young cultures of Pfeiffer's bacilli occasionally protected other rabbits against one fatal dose of homologous and heterologous filtrates, but no serum protected against more than one lethal dose of poison. Normal rabbit serum protected almost as many animals as did the sera of the inoculated rabbits. The nature of the poison remains in doubt, but it is evidently not a toxin, since there is no definite incu-

bation period between its inoculation and the appearance of symptoms, and a protective serum does not result from its repeated injection into rabbits.

Protection Experiments.

Attempts to protect mice against lethal doses of Pfeiffer's bacilli by means of rabbit sera made with poisonous filtrates proved unsatisfactory. The sera never reached a high potency because the rabbits became emaciated and died when the injected poison was a strong one, and they reacted but slightly to a weak one. Consequently the results of protection experiments on mice with such a serum were irregular and misleading. A strong antibacterial serum was more readily obtained and protected a greater number of mice against a lethal dose of Pfeiffer's bacilli, whether the serum was given 24 hours before the bacilli were injected, or after 15 minutes contact with the dose of culture (Table II).

TABLE II.
Protection Experiments with Mice.

No. of animals.	Serum (rabbit).	Method of administration.	Survived.
			<i>per cent</i>
53	Antitoxic (?).	24 hrs. before bacilli.	3.8
34	" (?).	After 15 min. contact with bacilli.	23.0
16	Antibacterial.	" 15 " " " "	25.0
20	"	24 hrs. before bacilli.	40.0
10	Normal.	24 " " "	1.0
10	"	After 15 min. contact with bacilli.	2.0

Human sera from six recovered patients were entirely lacking in protective effect when tested on mice inoculated with lethal doses of Pfeiffer's bacillus, although all these sera contained complement-binding bodies.

DISCUSSION.

As regards the serological reactions of Pfeiffer's bacillus with the sera of recovered patients, as well as with monovalent immune rabbit sera, the results with agglutination were irregular and not satisfactory.

As far as they showed positive results the epidemic and the sporadic strains reacted similarly.

At the Department of Health of the City of New York, Park and his coworkers⁶ found Pfeiffer's bacillus present in 80 to 100 per cent of influenza patients. The bacilli isolated from different cases, however, did not produce identical immune bodies in inoculated animals as measured by the agglutinin absorption test, and Park concludes from these studies that there were many strains of Pfeiffer's bacillus, and not one epidemic strain, present during the pandemic.

Complement fixation reactions with human sera showed that normal controls did not contain complement-binding bodies for the Pfeiffer bacillus, but that the blood of recovered patients gave a positive reaction with more or less regularity in dilutions varying from 1:5 to 1:20. The binding could almost always be obtained with antigens made from more than one strain of the bacillus. On the other hand, with the sera of two patients in whose sputum Pfeiffer's bacilli were not found, and who suffered from lobar pneumonia and laryngitis respectively, no fixation was obtained. It would seem, then, that the reaction is due to something more than the presence of an increased amount of non-specific protein after a febrile attack.

With some immune rabbit sera binding was obtained in dilutions of 1:100. Normal human and rabbit serum controls did not bind.

Rapoport⁷ examined a much larger series of cases than I was able to do. He studied influenzal pneumonia patients and controls. His results showed complement fixation bodies in 54.5 per cent of 295 convalescent influenzal pneumonia patients and in only 9.67 per cent of 300 controls. My results confirm his and show that patients who are convalescent from influenza without pneumonia also may have complement fixation bodies in their serum for a limited period of time and to a less marked degree.

Kolmer, Trist, and Yagle⁸ demonstrated complement fixation in the serum of convalescent influenza patients with antigens made from Pfeiffer's bacillus, hemolytic streptococci, and *Micrococcus catarrhalis* but not with staphylococcus and pseudodiphtheria bacillus antigens.

⁶ Park, W. H., *J. Am. Med. Assn.*, 1919, lxxiii, 318.

⁷ Rapoport, F. H., *J. Am. Med. Assn.*, 1919, lxxii, 633.

⁸ Kolmer, J. A., Trist, M. E., and Yagle, E., *J. Infect. Dis.*, 1919, xxiv, 583.

45 to 50 per cent of the sera reacted with Pfeiffer's bacillus, and only 38 per cent bound complement when streptococcus or *catarrhalis* antigens were employed. All the sera were tested in a dilution of 1:10. Consequently no light is thrown on the specificity of the reactions.

Precipitins were also found constantly in the sera of recovered patients and of immunized rabbits. The reaction for them was strongly marked both with heterologous and with homologous strains of Pfeiffer's bacillus.

CONCLUSIONS.

It has been shown that the sera of patients convalescent from influenza yield reactions for agglutinins, precipitins, and complement-binding bodies with antigens of Pfeiffer's bacillus. These reactions appear constantly at the end of the 1st week, increase in intensity during the 2nd week, and remain demonstrable for a period of 2 to 4 months. They were most complete in the sera of patients suffering from postinfluenzal pneumonia. It has also been demonstrated that the strains of Pfeiffer's bacillus isolated during the epidemic were morphologically and biologically similar to the strains isolated from influenza cases in other years, and antigenically they differed from them only quantitatively. The patients' serological reactions indicate the parasitic nature of the bacillus, but are not sufficiently stable and clean-cut to signify that Pfeiffer's bacillus is the specific inciting agent of epidemic influenza. They do, however, indicate that the bacillus of Pfeiffer is at least a very common secondary invader in influenza, and that its presence influences the course of the pathological process.

A STUDY BY THE SINGLE CELL METHOD OF THE INFLUENCE OF HOMOLOGOUS ANTIPNEUMO- COCCIC SERUM ON THE GROWTH RATE OF PNEUMOCOCCUS.

BY M. A. BARBER, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, July 15, 1919.)

Ascoli (1) attributed the immunity observed in certain experiments with anthrax to the antimetabolic action of immune serum on the bacilli, represented, in part, by the inhibition of capsule formation. He was the first to use the term antiblastic immunity in this connection. Dochez and Avery (2) found that the earlier growth of pneumococcus in homologous antipneumococcic serum was inhibited by the serum. They estimated the degree of inhibition by plating out samples and counting colonies. They reported a further inhibition of the metabolic activities of pneumococcus by homologous serum as measured by the production of amino-acids and by the fermentation of glucose, saccharose, lactose, and inulin. The serum of patients at different stages of pneumonia infection showed a like inhibitory action on the digestion of protein and the fermentation of inulin. Blake (3) repeated the experiments of Dochez and Avery and extended them. He obtained, in part, similar results, but put a different interpretation on the findings. Since pneumococcus tends to grow in chains and clumps in homologous serum, a sample of such growths would give fewer colonies than the controls, even when the numbers of bacteria were exactly the same. Further, the clumped masses of bacteria tend to collect in the bottom of the test-tube, and the lack of diffusion of formed products would explain the results of Dochez and Avery in regard to the fermentation of sugars and the production of amino-acids. In cultures frequently shaken or grown in shallow layers of medium in flasks Blake obtained the same metabolic activities in homologous serum as in controls. Homologous sera deprived of agglutinins gave the same results as heterologous sera. He confirmed the observation of von Dungern (4) that the liquefaction of gelatin by *Staphylococcus aureus* is inhibited by homologous serum, but, according to his observation this inhibition of liquefaction was not accompanied by a diminution of such metabolic activities as are essential to growth.

It seemed worth while to investigate further the possible inhibition of the growth of pneumococcus by homologous immune serum, with especial reference to the effect of the serum on the earlier genera-

tions, by a method which allows a comparatively accurate measurement of the growth rate of bacteria. The problem of infection and resistance is complex, involving factors the action of which is still obscure; and if one possible factor should be demonstrated and prove to be measurable, or if it should be definitely eliminated, we would have an advance in our knowledge of the mechanism of immunity.

In addition to accuracy in measurement of growth rate, the use of the single cell method offers other advantages in this problem. Similar sowings, the immediate progeny of a single cell, may be employed for the test culture and the controls. Agglutination may be eliminated, in the earlier generations, at least. Further, it is possible that the use of single or few cells in culture experiments may more closely simulate the process of infection in nature.

The method of single cell culture as described previously (5) was employed in these experiments with some improvements and minor modifications to suit the problem and the organism studied. No especial difficulty was encountered in working with pneumococci. They were easily isolated and grew readily, and multiplication in hanging drops proceeded apparently at the same rate as in the test-tube.

In practically all the experiments in hanging drops described in this paper, controls were made under the same conditions, so that it was not necessary to ascertain exactly the normal growth rate of pneumococcus. However, it was found that some approximation of this growth rate would be convenient, especially in estimating the degree of lag, when such occurred. Many tests showed that with Type I and Type II pneumococcus, grown in hanging drops under the best conditions—a favorable medium and temperature, and seeding material taken in an actively dividing stage—the growth rate approximates one generation in 30 minutes. In a few instances growth was apparently more rapid, possibly a generation every 25 minutes, but this occurred in only a small minority of the tests. Single pairs sown in test-tubes of 5 or 10 cc. of serum broth gave approximately the same rate. Here the number of cells in the culture was estimated by means of the counting chamber. It is hardly practicable to estimate the minimum generation time by means of plating out methods, since at the period of growth when the chains are breaking up, one

would get an apparent growth rate much more rapid than the real one. As the author has shown (6), colon bacilli also break up into much smaller elements at a certain period of growth, so that the plating out method with bacilli also is subject to the same error. In this paper the generation time of 30 minutes is taken provisionally as the normal rate for pneumococcus.

Experiments with Immune Horse Serum in Hanging Drops.

The method and results of hanging drop experiments are best illustrated by protocols of one or two experiments.

A single pair of *Pneumococcus* Type I, grown for some time on artificial media, was sown in a hanging drop of blood broth. After about five generations had formed, single pairs of the progeny of this pair were sown in hanging drops of homologous highly immune horse serum of Type I and also in drops of highly immune horse serum of Type II and of normal horse serum, all diluted 1:52 in nutrient broth. The growth was studied in five droplets of the diluted homologous serum, four of the heterologous serum, and two of the normal horse serum, all on the same large cover-glass and under like conditions. After 170 minutes incubation the cells in the droplets were killed and stained by injecting a stain through a capillary pipette under microscopic control. A special fixing and staining fluid, made by adding medicinal methylene blue to a solution of potassium hydroxide immediately before use, was employed. The number of pairs present was determined with a $\frac{1}{12}$ oil immersion lens, and the size of the organisms in the various droplets was compared and also compared with the size of the seeding pairs which had been noted at the time of inoculation.

In this experiment one pair in heterologous serum failed to grow. In all the remaining ten drops, including those containing the homologous serum, the amount of growth was almost exactly the same—slightly less than six generations—giving a growth rate of approximately 29 minutes. There was no greater tendency to lag in the homologous serum than in the controls.

In the experiment just described conditions were favorable and growth proceeded at a geometrical rate and at approximately the maximum rate for pneumococcus in hanging drops. In a second experiment the conditions were somewhat different. Undiluted serum was used with just enough nutrient agar added to make a thin jelly, two parts of agar to six of serum. In preparing the seeding material a single cell was isolated from a blood broth culture of *Pneumococcus* Type I, made from the blood of a pneumonia patient, and this cell was grown in a hanging drop of human blood broth to four generations. The cells formed adhered in a chain, and only four generations formed in $2\frac{1}{2}$ hours, indicating that the pair sown was not actively growing at the time of isolation. This chain was broken up and distributed in drops of homologous and heterologous sera as shown in Table I.

TABLE I.

Growth of Pneumococcus Type I in Agar Serum Jelly. Incubation 180 Minutes.

Type I serum.			Type II serum		
Drop No.	Sowing. Pairs.	No. of genera- tions formed.	Drop No.	Sowing. Pairs.	No. of genera- tions formed.
1	1	4	1	1	4
2	1	3	2	2	4
3	3	3	3	3	3

In this experiment growth evidently lagged and the cells stained irregularly, so that the number of generations could only be approximated, but there was apparently no greater inhibition of growth in the homologous serum than in the control.

The protocols given above are representative of many hanging drop experiments. Sera were used undiluted and in dilutions up to 1:52. Dilutions were made in normal salt solution as well as in plain broth, glucose broth, and agar. Seeding material was taken from cultures long grown on artificial media, from infected human blood, and directly from the heart's blood of mice that had died of pneumococcus infection. In some experiments seeding material was taken at the height of growth, in others from a 22 hour culture, and in others from cultures at various other periods of growth. Lag and irregular growth occurred in some instances, especially when the seeding material was old or when it was sown in undiluted or little diluted sera, but in all these experiments the controls showed the same degree of irregularity and inhibition of growth as occurred in the homologous serum. To sum up, the hanging drop experiments gave no evidence of a measurable inhibition of growth in homologous serum.

While the growth rate in homologous serum was apparently unaffected, the character of the growth was distinctly different from that observed in heterologous serum or in other controls. The cells early became invested with a thick capsule and grew in chains, which often intertwined and formed zooglea-like masses. It was sometimes necessary to disentangle these chains by means of a fine capillary point, in order to count the cells. The capsule formed also on cells which were isolated from old cultures and were apparently dead or, at all

events, subsequently showed no growth in hanging drops. When sputum containing pneumococcus was incubated in a mixture of broth and homologous serum, these zooglea-like masses were distinctly seen, suggesting a possible means of early recognition of types of pneumococcus in sputum. This type of growth can hardly be called agglutination in the strict sense of the word, but resembles zooglea formation. The thick capsules are probably concerned in the protection of the organism, and their formation may be analogous to the formation of capsules by many bacteria in the animal body.

Experiments with Immune Horse Serum in Test-Tubes.

If homologous immune serum causes no inhibition of the growth of pneumococcus during the first five or six generations, it is not likely that any inhibition would occur in later growth, except that in the later periods the clumping of the cells and localization in one part of the container might interfere with the free diffusion of nutrient material and waste products, and in this way interfere with the free growth of the cells. Nevertheless, it seemed important to investigate this matter, and experiments of the following type were performed. One or more pairs of pneumococci were inoculated into test-tubes containing 2 to 10 cc. of diluted sera. In the experiment given in Table II 1 per cent glucose broth was used to dilute the serum, since the clumps formed in homologous immune serum when diluted with this medium apparently disintegrate earlier than when the dilutions are made in plain broth, and the cells can therefore be more readily counted. In this experiment homologous and heterologous antipneumococcic sera were employed in a dilution of 1:26 in 2 cc. of broth. As a control, Tube 7, which contained 11 cc. of glucose broth plus 0.1 cc. of rabbit serum, was included. As seeding material we used the progeny of one pair of Type I pneumococci, grown for 2 hours in a hanging drop of rabbit serum glucose broth. At the end of this period sixteen pairs had formed. The cells were apparently in an active state of multiplication.

The number of bacteria was ascertained by counting the stained cells in a counting chamber. In Tube 1 chains and clumps appeared, so that only an approximate count could be made, but, since even an

error of 100 per cent in counting would make a difference of only one generation, the error in the estimated generation time must be small. The growth in Tubes 3 and 4 was allowed to continue until the medium had become densely cloudy and the clumps had largely disintegrated. A more accurate count could then be obtained. In Tubes 3, 4, and 7, the growth had begun to lag on account of crowding before the count was made, so that the generation time is apparently greater in these tubes. All the cultures were well shaken at intervals after about 12 hours growth. Tube 2, containing Type II serum, showed cloudiness before any other. Otherwise the growth was ap-

TABLE II.

Growth of Pneumococcus Type I in Glucose Broth. Homologous and Heterologous Immune Sera, 1:26 Dilution.

Tube No.*	Type of serum.	Sowing. Pairs.	Length of incubation before cloudiness appeared.	Length of time allowed to grow before counting.	No. of generations formed.	Generation time.
			hrs.	hrs.		min.
1	I	1 medium sized.	13 $\frac{3}{4}$	14 $\frac{1}{3}$	26.2	32.8
2	II	1 " "	12 $\frac{1}{4}$	14 $\frac{1}{3}$	27.4	31.4
3	II	1 " "	13	20 $\frac{1}{2}$	28.8	42.8
4	I	1 " "	13	20 $\frac{1}{2}$	29.1	42.4
5	II	2 small.	No growth.			
6	I	2 " "	" "			
7	Rabbit serum.	2 " "	16	18 $\frac{3}{8}$	30.2	34.3

* The tubes were inoculated in the order given.

proximately the same in all the tubes containing immune serum. In the two tubes containing homologous serum in which growth occurred, the clumping ordinarily observed in such serum was manifest.

In the following experiment (Table III) the homologous and heterologous sera were diluted 1:10 in plain broth and 5 cc. quantities were used. The seeding material, Type I pneumococcus, was taken directly from a broth culture at the height of growth. The cells were isolated and each was separately transferred to the serum dilutions in the test-tubes. Six hanging drop cultures in plain broth were also made at the same time with cells isolated from the same source. In five of these cultures two generations occurred during

the 1st hour of incubation, and in one, one generation. It is probable therefore, that all cells sown into the serum dilutions were viable and actively growing.

On account of the formation of large clumps in the homologous serum tubes, it was hardly practicable to estimate comparative amounts of growth either by counting or by the degree of cloudiness. In consideration of the smallness of the sowings, it is apparent that growth in the homologous serum suffered little if any lag. The number of successful growths in the homologous serum was nearly as great as that in the heterologous, three out of five in the one and four out of five in the other.

TABLE III.

Growth of Pneumococcus Type I in Plain Broth. Homologous and Heterologous Sera, 1:10 Dilution.

Tube No.*	Type of serum.	Sowing. Pairs.	Growth after 15½ hrs. incubation.
1	I	1	Clear above; clumps at bottom.
2	II	1	Cloudy ++.
3	I	1 small.	No growth.
4	II	1 “	“ “
5	I	1 “	“ “
6	II	1 “	Cloudy ++.
7	I	2 “	Clear above; clumps at bottom.
8	II	2 “	Cloudy ++.
9	I	4 “	Clear above; clumps at bottom.
10	II	4 “	Cloudy ++.

* The tubes were inoculated in the order given.

In another experiment 10 cc. lots of broth plus homologous serum, diluted 1:50 and 1:100, were employed, and the growth in these was compared with the growth in similar quantities of the same broth without any serum. Sowings of one and two pairs were made. There was less lag in the tubes containing serum than in the plain broth tubes, probably entirely due to the presence of the serum. Clumping occurred in the 1:100 dilution but in less degree than in the 1:50 dilution. Growth was visible macroscopically in both dilutions at the end of 15 hours, while the plain broth tubes were still clear 3½ hours later.

In short, the test-tube experiments show that growth in homologous serum proceeds at nearly, if not quite the same rate as that in the heterologous serum in spite of the formation of clumps.

Action of Homologous Serum Plus Complement.

In the experiments described above no fresh complement was added other than that contained in blood or serum broth. In order to test the effect of homologous serum plus complement on growth rate, preliminary experiments were arranged in which varying amounts of fresh rabbit blood, fresh human blood, or rabbit blister fluid containing more or less active leucocytes were added to homologous serum. In these mixtures the homologous serum gave no indication of a greater power to restrain growth of pneumococcus than the controls.

Experiments in Vivo.

Intravenous Injection of Immune Serum.—It was then proposed to ascertain whether homologous serum acquired some growth-inhibiting substance through contact with animal tissues *in vivo*.

A normal rabbit, weighing 3,200 gm., was given intravenously 5 cc. of a Type I immune horse serum. 1 hour later the rabbit was bled from the ear. Before coagulation had taken place, part of the blood was diluted in plain broth, one part of blood to four of broth, and another portion was collected in a centrifuge tube, in order to obtain serum for the tests to be described later. To serve as controls, blood of the same rabbit taken immediately before the injection of the serum was similarly diluted, and, in addition, a second control was made by adding 0.5 cc. of the normal rabbit blood to 9 cc. of broth containing 0.5 cc. of the same Type I serum that was inoculated into the rabbit. The tubes were immediately transferred to the ice chest. A series of hanging drop cultures was made at the same time in the 1:5 dilutions of normal blood, in the blood taken after the serum injection, and in the mixture containing normal blood, immune serum, and broth. The inoculation of these drops was made with cells of *Pneumococcus* Type I taken from a young broth culture. After 2 hours incubation the growth formed was stained on the cover-glass and the cells were counted under the $\frac{1}{12}$ oil immersion lens. The results are given in Table IV.

TABLE IV.

Growth of Pneumococcus Type I in Hanging Drops. Normal and Immune Rabbit Blood, Dilution 1:5 in Broth. Incubation 2 Hours.

Normal blood.			Blood after injection of immune horse serum.			Normal blood plus homologous immune horse serum.		
Drop No.	Sowing Pairs.	No. of generations formed.	Drop No.	Sowing Pairs.	No. of generations formed.	Drop No.	Sowing Pairs.	No. of generations formed.
1	1½	0	1	1	3	1	1	3
2	1	4—	2	2	3	2	3	3
3	2	4	3	2	3+	3	1	3+
4	2	3—	4	1½	0	4	2	3+
5	1	4+	5	1½	0	5	1	3+
6	3½	2+	6	1	1	6	1	3
7	2	3	7	1	4	7	1	2
8	1	3	8	1	1	8	½	0
9			9	1	3	9	2	3

It is seen in Table IV that growth took place in a large proportion of the hanging drops, averaging about three generations in 2 hours. A varying amount of lag is shown, due possibly to unevenness in the sowing material, but, in the aggregate, growth was practically the same, in all the groups. The tendency to capsule formation, characteristic of growth in homologous serum, appeared in all except the controls in normal blood. The experiment was repeated on the same day, with actively dividing cells of like ancestry grown on the cover-glass as sowing material. The same 1:5 dilutions in broth of blood taken before and after immunization were employed, but plain broth was substituted for the control with normal blood plus homologous serum. Growth proceeded at nearly the normal rate for hanging drop cultures—three to four generations in 90 minutes. The rate of growth in the blood of the animal which had received immune serum was practically the same as that in the blood of the normal animal.

On the following day a comparison was made of the undiluted serum of the rabbit taken before and that taken 1 hour after injection of immune serum. Seven hanging drops were made of each, and sowings were made from an actively growing broth culture. The serum was undiluted except through the small amount of moisture of conden-

sation present on the cover-glass. Plain broth controls were used. Growth occurred in all, with the exception of one broth control, but tended to lag in the undiluted serum. There was no measurable difference between the number of generations formed in the normal serum and in that obtained following the injection.

In order to compare the results obtained in hanging drops with those obtained in test-tubes an experiment was arranged with the same sera that were used in the last experiment. The sera were diluted in plain broth, 0.5 cc. of serum to 4.5 cc. of broth. Sowings were taken from a young plain broth *Pneumococcus* Type I culture, and, in order to get similar cells, one long chain of cocci was broken up to furnish the sowings. The results are given in Table V.

TABLE V.

Growth of Pneumococcus Type I in Test-Tubes. Normal and Immunized Sera, Dilution 1:10 in Broth.

Tube No.*	Rabbit serum.	Sowing. Pairs.	Growth after 15 hrs. incubation.	Growth after 19½ hrs. incubation.
1	After injection of immune serum.	2	Clear above with a clumped mass in the bottom.	Cloudy ++.
2	Before injection of immune serum.	4	Cloudy ++.	" ++.
3	After injection of immune serum.	2	Clear.	" ++.
4	Before injection of immune serum.	2	Cloudy ++.	" ++.

* The tubes were inoculated in the order given.

It will be noted in Table V that growth took place in all the tubes, with some lag in No. 3. However, in consideration of the variability of lag in test-tube experiments of this kind, whether immune serum is present or not, the lag in this tube can hardly be attributed to the presence of the homologous serum.

The experiments with the serum of the passively immunized rabbit were repeated with the serum of another similarly immunized animal, with especial attention to any inhibitory substance in the whole blood or undiluted serum. A 3,000 gm. rabbit was given intravenously 5 cc. of a potent Type I serum. 23 hours later a sample of blood was

drawn from an ear vein into a capillary pipette, and, before coagulation had taken place, was added to sowings previously isolated in minimum sized drops on the cover-glass. The sowings were taken from an actively growing rabbit blood broth culture of Type I pneumococcus. To serve as controls, a similar series of drop cultures was prepared on the same cover-glass with normal rabbit blood and rabbit blood broth. The results after $2\frac{1}{2}$ hours incubation are given in Table VI.

TABLE VI.

Pneumococcus Type I. Culture in Immunized Rabbit Blood, in Normal Rabbit Blood, and in Broth.

Blood of passively immunized rabbit.			Blood of untreated rabbit.			Broth.		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	$\frac{1}{2}$	5	1	1 small.	4+	1	$\frac{1}{2}$	5
2	$\frac{1}{2}$	5	2	1 medium sized.	4+	2	$\frac{1}{2}$	5
3	1 large.	5	3	" "	4+	3	$\frac{1}{2}$	5
4	$\frac{1}{2}$	5	4	" "	4+	4	$\frac{1}{2}$	0
5	1 large.	5+	5	$\frac{1}{2}$	5	5	$\frac{1}{2}$	5
6	$\frac{1}{2}$	5	6	2 large.	5	6	1 small.	5±
7	2 medium sized.	5+	7	2 medium sized.	4			

As shown in Table VI all the droplets showed growth except one of the broth, and growth occurred in all at approximately the normal rate. The cells in the immune blood formed chains characteristic of growth in homologous serum, but showed no lag as compared with the controls.

Similar series of cultures were made in the whole blood of the same rabbit taken $1\frac{1}{2}$ and 21 hours after the injection of serum. In neither series was there any evidence of inhibition of growth attributable to immune substances in the whole blood.

A series of test-tube experiments was then done with the whole blood, the serum, and the plasma of the immunized rabbit taken 3 days after the injection of the immune serum. Sowings of cells taken from an

actively growing Type I broth culture were made into the bottom of dry test-tubes. Into each tube only a single chain was sown, the chains varying in length from four to seven pairs each. Only sufficient broth to expel the chains from the pipette, amounting in each instance to a single small droplet, was discharged into the tubes. Three of the tubes so prepared received 1 cc. each of whole uncoagulated blood, not citrated; three received 2 cc. each of fresh serum; three, 2 cc. of fresh plasma; and three, 2 cc. of rabbit blood broth to serve as controls. The next day growth was evident in all except the three which received the whole blood. The growth appeared in the coagulated plasma in the form of single colonies in two of the tubes and as two colonies in the third. The growth in the serum and plasma was plated out and proved to be pneumococcus. The failure of growth in the whole blood might be attributed to phagocytosis, judging from the positive results obtained in hanging drops, but of this there is no definite proof.

On the same day the growth in plasma, in undiluted serum, and in whole blood from the same rabbit was compared with the growth in rabbit blood broth in hanging drops in much the same way as in the experiment described in Table VI. Growth failed in one of seven plasma droplets and in three of seven whole blood droplets. In the whole blood there was some evidence of phagocytosis in three of the droplets in which growth failed or was deficient. Growth occurred in all the serum droplets and in the broth controls. In all four sets when positive results were obtained, the amount of growth was approximately the same.

In summarizing all the experiments done with homologous serum, whole blood, or rabbit blood plasma *in vivo*, we find no measurable influence of the immune serum on the growth rate of pneumococcus.

Action of Homologous Serum in Vivo, in the Peritoneum of the Mouse.

In a series of experiments a study was made of the behavior of pneumococcus in the peritoneal cavity of normal and immunized mice. Mice were passively immunized by means of the injection of measured amounts of immune serum at varying periods prior to the injection of the bacteria. Sometimes the same mouse was used repeat-

edly for different experiments, so that it may have acquired some degree of active immunization by virtue of the repeated doses of bacteria. Cultures of different degrees of virulence and at different stages of growth were compared, and various sized doses of cocci were given. The object of the experiments was to ascertain whether the disappearance of the pneumococcus in the peritoneal cavity of the immunized mouse is due in any measure to the humoral activity of the peritoneal fluid. The protocols of several experiments will illustrate the method and results.

A mouse was given intraperitoneally 0.2 cc. of Type I antipneumococcic serum, and 1 hour later received 0.1 cc. of a rabbit blood broth culture of Type I pneumococcus, also intraperitoneally. The culture was the second transfer from the heart's blood of a mouse and had grown $15\frac{1}{2}$ hours at high room temperature. It was very cloudy and contained mostly discrete pairs or short chains. A test of viability was made at the time of inoculation by sowing single pairs of cells into hanging drops of broth. Twenty-six out of twenty-seven of these pairs grew, but the early growth was at only about half the normal rate. So practically all the cells inoculated were viable but not at the full height of growth.

At the same time that the immunized mouse was inoculated, a normal control received exactly the same dose of the same culture.

Samples of peritoneal fluid were taken from both mice by means of capillary pipettes 1 hour and 10 minutes, 4 hours, and 6 hours and 10 minutes after the inoculation of the bacteria. Additional samples were taken from the immunized mouse at about 8 hours (7 hours and 55 minutes) and at 28 hours after inoculation. In the control mouse numerous leucocytes and numerous cocci, mostly in separate pairs, were found at all periods. Phagocytosis, if present at all, was little marked. The mouse died during the night following the inoculation of pneumococci. In the immunized mouse many leucocytes and a few extracellular cocci, mostly in short chains, were found at the end of the 1 hour and 10 minute period. Some of these free chains were isolated into hanging drops of peritoneal fluid and grew at apparently the normal rate. Phagocytosis was marked, and the number of cocci found in phagocytes was apparently sufficient to account for the diminution of free cocci in the peritoneal fluid. At the end of the 4 hour

period and thereafter no extracellular bacteria could be found, although they were distinctly demonstrable in leucocytes at the end of 8 hours.

It was demonstrated that cocci contained within leucocytes of the immunized mouse were viable as late as 6 hours after inoculation and 2 hours after the period when extracellular cocci had apparently disappeared. This was proved by the following experiment.

Hanging drops made of the peritoneal fluid both undiluted and diluted in broth were allowed to incubate, and were then stained by means of a capillary pipette under microscopic control. These preparations were compared with similar preparations made from drops which were stained before incubation. In some leucocytes there was apparently no growth of cocci during incubation and the bacteria in some instances appeared to have degenerated. In others, small colonies had formed in and around the phagocyte. No evidence of bacteriolysis was found which could be ascribed to the effect of the homologous serum. A few free bacteria stained irregularly, but in no larger proportion than may be found in normal serum.

Hanging drop and plate cultures of peritoneal fluid were in all cases compared with films on slides subsequently stained by Gram's method and with Manson's methylene blue. The immunized mouse remained alive and well 6 days after the experiment.

In another experiment extracellular cocci taken from the peritoneal fluid of an immunized mouse were isolated in hanging drops containing a mixture of peritoneal fluid and broth. These cultures showed growth at fully the normal rate, five generations in $2\frac{3}{4}$ hours. In this case the mouse had received 0.2 cc. of homologous serum, followed by 0.05 cc. of a culture of *Pneumococcus* Type I, the first transfer from the heart's blood of a mouse. Tests in hanging drops had shown that practically all the bacteria inoculated were viable. This mouse had received no injection of bacteria or serum previous to the day of the experiment. The peritoneal fluid for the test was removed 35 minutes after injection of the bacteria. Phagocytosis was marked. After 1 hour and 25 minutes the fluid contained no free bacteria.

Growth Rate in Vivo in the Peritoneal Fluid of the Passively Immunized Mouse.

As noted in the previous experiments non-phagocytosed pneumococci isolated from the peritoneal cavity of the mouse grow in hanging drops at the normal rate. It was next proposed to ascertain whether growth takes place *in vivo* in the peritoneal fluid and to attempt to determine approximately the rate of growth, advantage being taken of the tendency of Pneumococci Type I to adhere in chains in homologous serum. These experiments were suggested by the observation that extracellular pneumococci found after 1 or 2 hours in the peritoneal cavity were usually in chains. One protocol will illustrate the method and results.

A mouse was given intraperitoneally 0.2 cc. of Type I serum. About 1½ hours later it was given a dose of Type I culture. The inoculum was prepared as follows: A rabbit blood broth culture was incubated until it became well clouded and examination of hanging drops showed that most of the chains had broken up. A portion of the culture was then centrifuged at low speed for about 5 minutes, in order to throw down the chains. The supernatant fluid was nearly clear and hanging drop examination showed that no long chains remained. A series of cells was isolated in hanging drops and all proved viable and actively dividing. 0.4 cc. of the supernatant fluid was inoculated intraperitoneally. A sample of peritoneal fluid was taken in about 2 minutes, a second sample 60 minutes later, and further samples at 30 minute intervals. The samples were placed on slides with little spreading, dried, fixed in alcohol, and stained with Manson's methylene blue. The incidence of pneumococci in pairs or in chains of various lengths was noted in several fields taken at random. The results of the countings are given in Table VII.

Samples taken at 150 minutes and later showed nearly all the chains either in phagocytes or broken into irregular groups. Up to the fourth sample the chains were fairly intact, owing to the capsular material which held them together. A few chains in the fourth sample had begun to break up. Phagocytosis became marked at 90 minutes, bacteria became fewer, and an increasing proportion of the contents of the phagocytes consisted of longer chains. Few single pairs were found at the end of 60 minutes. The mouse recovered.

By comparing the incidence of chains in the different samples in Table VII it becomes evident that growth was going on at a rate of about one generation in 30 minutes. In the first sample most of the cocci were in single pairs, with the next greatest number in chains of two pairs. 60 minutes later the greatest number of cocci were in chains of 4 and 4 to 8 pairs. 30 minutes later the largest number of cocci were in chains of 8 and 8 to 16 pairs. This indicates that the rate of division in the peritoneal cavity approximates the usual rate observed in hanging drop cultures.

TABLE VII.

Length of Chains of Pneumococci in Samples of Fluid Recovered from the Mouse Peritoneum.

Sample No.	Length of time after inoculation, <i>min.</i>	No. of pairs of cocci per chain.									
		1	2	2-4*	4	4-8	8	8-16	16	16-32	32
1	2	94	41		4						
2	60	1	2	3	31	10	5	1			
3	90					6	25	17		1	
4	120	†					3	7	23	11	9

* The headings 2-4, 4-8, 8-16, 16-32 refer to chains some members of which were dividing to form chains of a higher order.

† Some single pairs occur in clusters, probably from broken chains. Three 3 pair chains, four 6 pair, and six irregular chains are not included.

A parallel experiment with Type I pneumococcus was done on a mouse which had received during the 10 days previous to the experiment three doses approximating 0.05 to 0.1 cc. each of broth culture of pneumococcus together with homologous serum. These inoculations had been made in connection with other experiments and some active immunity may have been conferred on the mouse. This mouse was passively immunized on the day of the experiment and almost exactly the same procedure was followed as in the experiment described in Table VII. The results were similar except that in this case the chains held intact longer and could be counted at the end of 205 minutes, when chains of 64 to 128 could be found. About the same growth rate was observed as in the previous experiment. This mouse also recovered.

Similar experiments were also done with *Pneumococcus* Type II. This type proved less favorable, since the chains lost their identity earlier and tended to fuse into masses, owing to the more abundant formation of capsular material. In one experiment, however, three samples taken at about 30 minute intervals gave similar results to those of the Type I experiments. In this experiment only 0.2 cc. of supernatant culture fluid was given. Phagocytosis began in about 30 minutes and continued actively. However, this mouse died 4 days after inoculation with pneumococcus.

Apparently the growth of extracellular pneumococci in the peritoneal cavity of the immunized mouse continues until the chains or groups are overtaken by phagocytosis, and the growth of some portion, at least, of the cells continues at a geometrical rate. A few chains were noted in which a part of the members failed to take the stain deeply. This can hardly be considered as due to the action of the immune serum, since a similar phenomenon is sometimes observed in chains formed in undiluted normal blood or serum. At all events, the proportion of such degenerated cells in the mouse peritoneum is too small for us to ascribe much importance to this phenomenon as a factor in the disappearance of the bacteria.

Capsule formation was marked in the peritoneal cavity of the immunized mouse, but in Type I pneumococcus infection this apparently interfered little with phagocytosis. In Type II infection groups of bacteria formed thick gelatinous masses, which may have offered some mechanical hindrance to the phagocytes, but there is no proof in these experiments that these capsules were an important element in the protection of the bacteria.

DISCUSSION.

The studies which have been described, in which the single cell method of cultivating bacteria was employed, have not been successful in demonstrating that immune antipneumococcic serum owes any of its effect to any power of inhibiting the growth of pneumococci. While it is still possible that the serum may have such an effect, it has not been possible to demonstrate it by the method employed. That antipneumococcic serum has a high protective and curative

value has been demonstrated, but the mechanism of this action is still not entirely clear. The attempts to analyze its mode of action have so far only succeeded in showing that it has the power of causing marked agglutination of homologous organisms and of changing the organisms so that they undergo phagocytosis. The work of Bull (7, 8) shows that the agglutination of bacteria *in vivo* is probably a more important phenomenon in immunity and recovery than was formerly believed. The fact that the protective power of any lot of antipneumococcic serum, as tested *in vitro*, is not always proportional to its agglutinating power is opposed to the assumption that agglutination is of paramount significance. The exact importance of the property of facilitating phagocytosis has also not been thoroughly determined. The fact that the only real test of antipneumococcic serum is its action *in vivo*, in protecting the life of infected animals, seems to indicate that its effect depends, in part at least, on properties which are only manifested *in vivo* and the nature of which are not yet fully understood.

CONCLUSIONS.

1. Under the conditions stated pneumococci grow as readily in the serum of horses highly immunized to the homologous organism as they do in normal horse serum, and the rate of growth is not appreciably diminished.
2. This failure of immune serum to affect the growth rate is not altered when fresh rabbit blood, fresh human blood, or rabbit blister fluid is added in order to supply any hypothetical complement which might be lacking.
3. We have not been able to show that when immune horse serum is injected intravenously into rabbits or intraperitoneally into mice, it acquires the property of killing pneumococci or inhibiting their growth.
4. Experimental evidence has been obtained indicating that in the peritoneal cavity of the passively immunized mouse the growth of extracellular pneumococci continues at apparently the normal rate, until the bacteria are engulfed by phagocytes.
5. The immunizing and protective power of antipneumococcic serum probably depends, in part at least, on properties which are not

at present known. It has not been possible in the present study to demonstrate that one of these properties consists in delaying the growth of pneumococci.

BIBLIOGRAPHY.

1. Ascoli, A., *Centr. Bakteriolog., 1te Abt., Orig.*, 1908, xlv, 178.
2. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1916, xxiii, 61.
3. Blake, F. G., *J. Exp. Med.*, 1917, xxvi, 563.
4. von Dungern, *Centr. Bakteriolog., 1te Abt.*, 1898, xxiv, 710.
5. Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.
6. Barber, M. A., *J. Infect. Dis.*, 1908, v, 379.
7. Bull, C. G., *J. Exp. Med.*, 1915, xxii, 484.
8. Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 25.

ANTIBLASTIC PHENOMENA IN ACTIVE ACQUIRED IMMUNITY AND IN NATURAL IMMUNITY TO PNEUMOCOCCUS.

By M. A. BARBER, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, July 15, 1919.)

In the preceding paper (1) the results are reported of an investigation of the power of the serum of highly immunized horses to inhibit the growth rate of pneumococci. In that investigation, in which the so called single cell method of cultivating organisms was employed, it was impossible to demonstrate any growth-inhibiting action of the serum. This was true even when the serum was placed in the peritoneal cavity of the mouse and under conditions in which its full protective action was being manifested. Nevertheless, it seemed important to exclude the possibility that while no inhibiting action by serum can be demonstrated, the whole blood when freshly drawn from the actively immunized animal may have an effect of this kind. It seemed possible that the so called antiblastic immunity might play a part in active immunity, although the serum of the actively immunized animals might exert its effect in producing passive immunity in an entirely different manner. Consequently, a series of experiments was undertaken to determine whether or not blood or plasma fresh from the immune horse had any effect on the growth rate of pneumococcus. The same single cell method of study employed in the previous investigation was used in this work.

A virulent strain of Pneumococcus Type II was used in all the tests, and the blood was furnished by a horse which had received many injections of the same strain and of various atypical Type II strains as well. The degree of immunity was such that 0.2 cc. of serum protected mice against 10,000 lethal doses of the Type II strain employed in these experiments.

Tests with Whole Fresh Blood and Fresh Serum Diluted in Broth.

In the first series of experiments broth dilutions of whole blood and fresh serum were compared with fresh normal horse serum controls. Both hanging drop and test-tube series were done, the latter with sowings of chains of one to three pairs into quantities of medium varying from 4 to 10 cc. The whole blood was diluted 1:11 and the serum 1:5. In all the tests growth occurred in the dilutions of the immune blood as well as in the controls. The growth rate, as shown by growth in hanging drops, was practically the same in the immune blood and serum as in the controls.

Tests with Whole Citrated Blood.

The citrated blood was used on the day of collection, with normal horse serum as a control, both undiluted. The normal horse serum had been kept some months in the ice chest. The results after 2 hours incubation are given in Table I.

TABLE I.

Growth in Hanging Drops of Whole, Undiluted Citrated Blood of an Actively Immunized Horse Compared with That in Hanging Drops of Undiluted Normal Horse Serum.

Pneumococcus Type II.

Immune citrated blood.			Normal horse serum.		
Drop No.	Sowing. Pairs.	No. of generations formed after 2 hrs.	Drop No.	Sowing. Pairs.	No. of generations formed after 2 hrs.
1	2 small.	4	1	1 medium sized.	3+
2	2 "	4	2	2 small.	3+
3	1 medium sized.	4+	3	1 large.	4—
4	2 " "	4+	4	1 medium sized.	4—
			5	1 large.	4

There was therefore no evidence in these experiments that the whole fresh citrated blood from the immunized horse caused any delay in the growth rate of homologous pneumococci. Further experiments were made with fresh citrated blood in test-tubes, diluted and undiluted, but no definite evidence of inhibitory action could be obtained.

It was then determined to test the effect of whole undiluted blood transferred directly from the ear of the horse to sowings of one and two pairs of pneumococci previously isolated on the cover-glass, the method previously described being followed. Fresh normal horse blood was used as a control. After 3 hours incubation the greatest amount of growth in any droplet was two generations, and few showed that much. The amount of growth in the blood of the immunized horse and in the control was about the same.

Many hanging drop experiments were also done with the fresh undiluted coagulated plasma of both the normal and the immunized horse. In obtaining plasma for these experiments, blood contained in centrifuge tubes was placed on ice immediately after draining from the animal. The uncoagulated blood was centrifuged free from cells and kept cold until ready for use. The plasma coagulated soon after exposure to higher temperatures, but remained fluid long enough to allow mixing with organisms previously isolated in test-tubes or on the cover-glass. In every case without exception growth either failed or was limited to one generation after $2\frac{1}{2}$ hours of incubation. There was rarely, if ever, any trace of bacteriolysis, however, and the organisms sown could usually be found after incubation. Broth controls of the seeding material grew well. The effect of coagulated plasma was also tested by mixing it in amounts of 2 cc. in the bottom of test-tubes with minute droplets of broth containing six to eight pairs of pneumococci. Growth almost always occurred both in the tubes containing plasma from the immunized horse and in those containing plasma from the normal horse. There was some evidence that growth under these conditions tends to lag, however, but this effect was observed both in the plasma of the immune and in that of the normal horses. Similar experiments were done with the fresh undiluted serum of the same normal and immune horses. Here too it was found that in hanging drops a marked prolongation of generation time occurred which was evident both in the normal and in the immune serum.

These experiments indicate that under the conditions described whole fresh horse blood and coagulated plasma, and also serum, are not favorable media for the growth of pneumococci. They do not

indicate, however, that this fact is at all related to the phenomena of immunity, since it is exhibited in both immune and normal horse blood.

Natural Immunity.

While the previous experiments have not afforded any evidence that inhibition of growth of pneumococci by serum or body fluids plays any part in acquired immunity, it is possible that natural immunity against pneumococcus infection may depend to some extent upon this property of the serum. The inhibiting effect of whole horse blood upon the growth of pneumococci might give some support to this view. The relative susceptibility of the horse to pneumococcus infection is not accurately known, but it is not generally considered to be very high as compared with that of the mouse or rabbit, for instance. Certain experiments previously performed (1), while not planned with this point in mind, give strong evidence that whole rabbit blood, unlike horse blood, does not have an inhibiting effect upon the growth of pneumococci.

Heist, Solis-Cohen, and Solis-Cohen (2) have reported that by a special method which they have devised, they have demonstrated a bactericidal action in the blood of chickens and pigeons, animals which are known to be highly immune to pneumococcus infection. Strouse (3), who studied the question of natural immunity of the pigeon, was unable to demonstrate any bactericidal action of pigeon serum and presented evidence to show that this immunity was due to the normal high temperature of the pigeon which was inimical to the growth of pneumococci. Kyes (4) also found no evidence of pneumococcal action of pigeon serum *in vitro*.

Heist, Solis-Cohen, and Solis-Cohen studied fresh whole pigeon blood. They used small glass pipettes, and sowings consisted of the small numbers of bacteria which adhered to the walls after the pipettes had been filled and emptied from broth cultures of pneumococci. The fresh blood is then brought directly into contact with these bacteria by sucking the blood into the tube. The tubes are sealed off, incubated, and the contents later blown on glass slides and stained. It is stated that by this method, with not only the undiluted broth cultures, but dilutions of the broth cultures as well for seeding, it is possible to ascertain whether bactericidal action has occurred and to what degree. They state that with this method they have demonstrated that pneumococci fail to multiply in pigeon blood. Chicken blood is also bactericidal but less so than pigeon blood, while normal rabbit blood and mouse blood have no such action. Immune rabbit

blood, however, has been shown in this way to have a specific bactericidal action, and they state that they have also demonstrated this action in the blood of recovered patients.

The method employed by these writers is, in our opinion, open to certain objections, and it seemed important to investigate this matter further, since it has a direct bearing on our problem.

In our experiments the hanging drop method with small sowings was employed. A minimum of broth was used in the isolation of the organisms, in some instances scarcely more than that of the volume of several erythrocytes. A record kept of the relative amount of broth employed showed that there was apparently no greater amount of

TABLE II.

Growth in Hanging Drops of Fresh Citrated Pigeon Blood Compared with That in Hanging Drops of Rabbit Blood.

Pneumococcus Type I.

Rabbit blood.			Pigeon blood.		
Drop No.	Sowing. Pairs.	No. of genera- tions formed.	Drop No.	Sowing. Pairs.	No. of genera- tions formed.
1	1 large.	3+	1	1 large.	2
2	1 "	3+	2	1 "	2-
3	1 "	3±	3	1 "	2-
4	1 "	3	4	1 "	2-
5	1 "	3+			

growth in the drops which were intentionally given a slightly larger amount of broth. After the isolations had been made, a relatively large drop of blood was added to each seeding in such a way that the blood was caused to engulf the minute droplet containing the isolated bacteria. By this method the behavior of an exactly known sowing could be followed under conditions in which any humoral factor might act, but in which there was a minimal chance of any phagocytosis occurring without being observed.

In two series of experiments fresh citrated undiluted pigeon blood was used. A protocol is given in Table II. A Type I pneumococcus culture, apparently somewhat past the maximum height of growth, was used for seeding. The time of incubation was 2 hours. Growth

occurred in all the droplets in both samples with a tendency to lag in both. The growth in the pigeon blood was about one generation behind that in the rabbit blood. The cocci tended to form chains in the pigeon blood and discrete elements in the rabbit blood.

A second experiment with virulent Type II culture gave essentially the same results.

In another experiment non-citrated blood taken from another pigeon was used. The organisms were isolated in a minimum of broth as before. Fresh non-citrated rabbit blood was used as a control. The animals were brought to the microscope and the blood was transferred by means of a capillary pipette to the droplets before coagulation had occurred. In one series the droplets were incubated for 5 hours in order to ascertain whether growth in the blood would continue beyond the first three or four generations. Actively dividing Type I cells taken from a broth culture were used for the sowings, which consisted of one pair or of a chain of two very small pairs per droplet. Of five droplets of rabbit blood all showed growth and gave approximately nine generations each. Of five droplets of pigeon blood, four showed growth, with approximately seven, six, two, and six generations respectively, a lagging of two to seven generations behind the growth in rabbit blood. The cocci in the rabbit blood tended to form groups of discrete elements, while those in the pigeon blood were mostly in chains.

Other experiments were made with the non-citrated fresh pigeon blood, and in these practically no difference appeared between the growth in rabbit and pigeon blood. A protocol of one of these is given in Table III. Sowings were taken from an actively dividing Type I culture, the same strain that was used in the previous experiments, and the time of incubation of the droplets was $2\frac{1}{2}$ hours.

Here the amount of growth was essentially the same in both blood samples, and in most droplets multiplication went on at the normal rate. In the pigeon blood there was a greater tendency to chain formation, and in the two droplets in which growth was deficient there was some evidence of phagocytosis.

In summarizing these experiments we may say that growth occurred about as often in the pigeon as in the rabbit blood, and the rate of growth in both was practically the same. By the method employed by

us, therefore, we were unable to demonstrate that pigeon blood has any bactericidal action or inhibiting effect on the growth of pneumococci. It is possible that the differences between our results and those of Heist, Solis-Cohen, and Solis-Cohen are due to the differences in the conditions of the experiment. However this may be, we have, by the technique employed, been unable to show any relation between natural immunity in the pigeon and inhibition of growth of pneumococci by the whole blood.

TABLE III.

Growth in Hanging Drops of Non-Citrated Pigeon Blood Compared with That in Hanging Drops of Rabbit Blood.

Pneumococcus Type I.

Rabbit blood.			Pigeon blood.		
Drop No.	Sowing. Pairs.	No. of genera- tions formed.	Drop No.	Sowing. Pairs.	No. of genera- tions formed.
1	$\frac{1}{2}$	5	1	$\frac{1}{2}$	4+ or 5
2	$\frac{1}{2}$	5	2	$1\frac{1}{2}$	2±
3	$\frac{1}{2}$	0	3	$\frac{1}{2}$	5
4	1 small.	5±	4	1 large.	5
5	$\frac{1}{2}$	4+	5	$\frac{1}{2}$	5
6	$\frac{1}{2}$	4+ or 5	6	1 small.	5
			7	1 "	3 or 4

SUMMARY AND CONCLUSIONS.

The paper gives the results of a series of experiments made with a special technique for the purpose of testing whether or not so called antiblastic phenomena or bactericidal phenomena play any part in immunity to pneumococcus. The technique employed is that devised by the writer and consists in the isolation of single bacteria and their growth in hanging drops where growth can be continuously observed. From these experiments it may be concluded that: (1) Whole fresh blood, coagulated plasma, or serum of the immunized horse added directly to pneumococci, has considerable inhibiting action on the growth of pneumococci. The inhibiting effect of the fresh blood, coagulated plasma, or serum of a normal horse, however, is as marked as that of a horse highly immunized to pneumococci of the same type.

This property, therefore, does not seem to be of importance in acquired immunity in the horse. No such property has been demonstrated in whole fresh rabbit blood. (2) It has been impossible to demonstrate that antiblastic phenomena play any part in natural immunity to pneumococcus, at least as far as the immunity of the pigeon is concerned. The whole fresh blood of the pigeon under the conditions employed exhibits no inhibiting action on the growth rate of pneumococci.

BIBLIOGRAPHY.

1. Barber, M. A., *J. Exp. Med.*, 1919, xxx, 569.
2. Heist, G. D., Solis-Cohen, S., and Solis-Cohen, M., *J. Immunol.*, 1918, iii, 261.
3. Strouse, S., *J. Exp. Med.*, 1909, xi, 743.
4. Kyes, P., *J. Infect. Dis.*, 1916, xviii, 277.

BLOOD VISCOSITY.

I. CONDITIONS AFFECTING THE VISCOSITY OF BLOOD AFTER WITHDRAWAL FROM THE BODY.

By LOVELL LANGSTROTH, M.D.

(From the Laboratories of the University of California Hospital, San Francisco.)

(Received for publication, July 22, 1919.)

Determinations of blood viscosity were taken up in this laboratory in connection with work on cyanosis and were found to be influenced by so many factors hitherto unconsidered that a more thorough investigation of the subject seemed warranted.

Viscosity may be defined as the internal resistance of the individual molecules of a liquid to passage over each other. It depends on the nature of the liquid and on the temperature. When a liquid flows through a capillary which is wet by it the movement of the layer of molecules next to the wall is nil and not dependent on the nature of the wall, while the movement in the center of the stream is the greatest.

According to Poiseuille (1), the volume of a liquid passing through a capillary tube is directly proportional to the 4th power of the diameter, the time, and the pressure, and inversely proportional to the length of the tube.

$$Q = \frac{d^4 p T}{l}$$

He showed that for each liquid there was a constant which was the reciprocal of the coefficient of viscosity for that liquid.

By the proportion

$$Q : \frac{d^4 p T}{l} :: k : \frac{1^4 \cdot 1 \cdot 1}{1}$$

the constant for the liquid is obtained

$$k = \frac{Ql}{d^4 p T}$$

and can be introduced into the equation thus

$$Q = k \frac{d^4 p T}{l}$$

by means of which the quantity Q can be determined for any capillary tube and pressure.

Since η , the coefficient of viscosity, is the reciprocal of k

$$\eta = \frac{d^4 p T}{Q l}$$

When a liquid flows through a vertical capillary under its own pressure,

$$p = h \cdot s$$

in which h is the height of the column and s the specific gravity of the liquid.

If we want merely the relative value of η we need only determine under similar conditions the outflow time of similar volumes.

$$\eta : \eta_1 :: \frac{T d^4 h s}{l} : \frac{T_1 d^4 h s_1}{l} = \frac{T s}{T_1 s_1}$$

$$\eta = \eta_1 \frac{T s}{T_1 s_1}$$

or since $\eta_1 = 1$

$$\eta = \frac{T s}{T_1 s_1}$$

If the specific gravities of the liquids under investigation are not widely different

$$\eta = \frac{T}{T_1}$$

when T_1 is the outflow time of distilled water.²

Method.

The specific gravity of most blood falls between 1,040 and 1,060, an average of 1,050. No attention has been paid in this work to the specific gravity of the blood, the viscosity simply representing the relation between the values of T for the blood under investigation and distilled water.

A viscosimeter of the type advocated by Determann (2) was made by a local glass-blower and, while differing from the original in having a capillary of slightly larger bore, gave satisfactory results as the value 0.073 minute for distilled water was repeatedly obtained.¹ The instrument requires only 0.2 cc. of blood for each determination and

¹ For a critical review of the different types of viscosimeters and the principles involved see Determann (8)

is provided with a water jacket and thermometer by means of which any desired temperature may easily be obtained. All determinations were made at 20°C. with a stop-watch registering to 0.001 minute, the instrument being cleaned and dried each time.

For work of the type planned it was impossible to use blood without some means of preventing coagulation. Defibrination, on account of the considerable changes it brings about (3), was not considered, and hirudine was not available. It has always been considered that the addition of oxalate increases the viscosity of the blood (3), but the work here reported shows that this change is apparently due either to insufficient mixing of the blood or to changes in its gas content. Small quantities of potassium oxalate were therefore used to prevent coagulation. Blood was withdrawn from an arm vein with a Record syringe and oxalated in various ways. At first the blood was immediately added to a small glass cup with a capacity of about 1 cc. containing a few grains of powdered oxalate. Later powdered oxalate was placed in the barrel of the syringe before drawing the blood, and finally the most satisfactory method was found to be that of wetting the inside of the syringe with a saturated solution of oxalate.

EXPERIMENTAL.

Effect of Oxalate on the Blood.

Experiment 1.—Blood was taken from an arm vein, put immediately into the small glass cup, the viscosimeter filled to the mark by suction from its opposite end, and the outflow time determined. Without removing the needle from the vein, more blood was taken into a Record syringe containing a little powdered oxalate. After considerable rotation to dissolve the oxalate, the viscosimeter was filled from the glass cup with this blood. The figures below show the results.

	Viscosity.
Unoxalated blood.....	7.15
Oxalated "	7.76

Experiment 2.—Blood was taken from an arm vein into a Record syringe wet with a 20 per cent solution of oxalate. The viscosimeter was immediately filled from the nozzle of the syringe and the outflow time determined. The remainder of the blood was put into a test-tube where it did not coagulate. Blood was taken as soon as possible from the same vein into another Record syringe wet with 0.9 per cent salt solution. The viscosimeter was immediately filled from the nozzle of the syringe and the viscosity determined.

	Viscosity.
Oxalated blood.....	7.02
Unoxalated "	7.02

Experiment 3.—Experiment 2 was repeated, but in filling the viscosimeter with the unoxalated blood a bubble of air entered the capillary. This had to be expelled and the instrument was refilled, with a consequent wait of a few moments. The delay was not sufficient to result in coagulation of the blood before the determination was made.

	Viscosity.
Oxalated blood.....	6.49
Unoxalated "	6.69

In Experiment 2 it will be noticed that there was no opportunity for change in the gas content of the blood and no chance for settling of the formed elements, as the viscosimeter was filled immediately from the nozzle. At the same time oxalate was present in sufficiently large amounts to prevent coagulation of the blood later in a test-tube. In Experiment 1 a few moments were spent in rotating the syringe to dissolve the oxalate. This gave opportunity for uneven distribution of the corpuscles and a large bubble of air due to the presence of dry oxalate in the syringe perhaps slightly changed the carbon dioxide content. These two factors resulted in a higher value for the oxalated blood. In Experiment 3 the viscosity of the unoxalated blood was greater, due evidently to settling of the corpuscles while the viscosimeter was emptied and refilled. It cannot be denied that when blood is oxalated in the manner described in Experiment 2 and the viscosimeter filled immediately from the nozzle of the syringe, there is not time for the blood and oxalate to mix thoroughly, and on this account the objection might be raised that in Experiment 2 the figure did not represent the viscosity of oxalated blood. It did not seem possible to overcome this feature in any way which would secure a uniform suspension of the red cells and a gas pressure the exact equivalent of that in the veins.

Effect of Different Quantities of Oxalate on the Same Blood.

Experiment 4.—15 cc. of blood were taken from an arm vein and 5 cc. put immediately into each of three separating funnels of 300 cc. capacity containing respectively 0.2, 0.4, and 0.6 gm. of dry powdered oxalate. Each funnel was im-

mediately stoppered, filled with cooled alveolar air,² and rotated for 1 minute. This operation was repeated three times or until no further increase in redness was observed. Each funnel was again carefully rotated for 1 minute in a horizontal position but with a constant slight alternate raising of each end, and blood was then drawn into a small glass cup for the viscosity determination by turning the stop-cock at the lower end.

	Viscosity.
Funnel 1 (0.2 gm. of oxalate).....	7.26
“ 2 (0.4 “ “ “).....	7.26
“ 3 (0.6 “ “ “).....	7.26

It seems clear from this that, other conditions being the same, the blood viscosity is not affected by varying amounts of oxalate. With the evidence obtained here and in Experiment 2 it is probably justifiable to conclude that small quantities of potassium oxalate do not alter the viscosity at all.

Effect of Insufficient Mixing of the Blood.

A very uniform suspension of red cells is necessary if correct viscosity determinations are to be made. This is shown by Experiment 5.

Experiment 5.—20 cc. of blood were taken from an arm vein into a Record syringe wet with a saturated solution of oxalate, and the viscosity was determined almost immediately by filling the instrument directly from the nozzle of the syringe. At frequent intervals afterward the blood in the syringe was mixed by rotating the syringe so as to move about a bubble of air which had been allowed to remain in it and the viscosity determined by filling the instrument from the nozzle. After again mixing the remaining blood as thoroughly as possible in the syringe, one-half was placed in each of two separating funnels, which were immediately stoppered and rotated for 1 minute in the manner already described. Blood was then taken from each by the stop-cock and the viscosity determined.

² In order to keep the carbon dioxide content of the blood about the same as that of the body, blood was often saturated with alveolar air which had been cooled to room temperature by passage through a vessel full of glass beads. As is shown in Paper II, this procedure sometimes actually increased the carbon dioxide content.

<i>a, m.</i>	Viscosity.
9.58 (immediately).....	6.85
10.05.....	7.0
10.08.....	6.30
10.15.....	6.03
10.20.....	6.52
10.25.....	6.60
Funnel 1.....	6.90
“ 2.....	6.96

The wide variation in viscosity in the first portion of the experiment was apparently entirely due to insufficient mixing of the blood as is well shown by the second portion. The viscosity after rotation in the separating funnel was slightly higher than the first and probably correct value, but this, as will be shown later, is to be expected from the slight loss of carbon dioxide.

Since the viscosity of the blood is affected by changes in its gas content, it was found convenient to secure a uniform suspension of the corpuscles by rotating the blood in a stoppered separating funnel, which at the same time insured an equilibrium between the gas content of the blood and the air in the funnel. Duplicate determinations were obtained without changing this equilibrium by opening the stopcock and drawing a small amount of blood into a 1 cc. glass cup. The time consumed in this manner before filling the viscosimeter did not seem to be sufficient to allow sedimentation of the blood.

Effect of the Amount of Blood.

The amount of blood in the funnel seemed to influence the results, as is shown by Experiment 6.

Experiment 6.—3 cc. of blood were placed in a separating funnel, stoppered, and then saturated with cooled alveolar air. The viscosity was then determined at intervals after carefully rotating the funnel each time for 1 minute.

	Viscosity.
Immediately.....	7.12
After 10 min.....	7.4
“ 30 “.....	7.68
“ 60 “.....	6.99

When the results of Experiment 6 are compared with those in Experiment 5 it is evident that at least 5 cc. of blood must be used to insure accurate results.

Effect of Standing in Contact with the Air.

It has already been mentioned that the viscosity was found to be quickly affected by change in the gas content of the blood. Oxalated blood when left standing in contact with room air rapidly loses carbon dioxide and diminishes in bicarbonate content, as has been shown by Van Slyke and Cullen (4). If the blood is covered with a thin layer of paraffin oil this loss is greatly diminished in rate, or if the vessel containing the blood is tightly stoppered carbon dioxide is given off until an equilibrium is reached between the pressure of carbon dioxide above and that in the blood. Ewald (5) has noted that the viscosity of oxalated blood increased with the time after withdrawal. Experiments 7 to 9 show the effect on viscosity of allowing the blood to stand in an open vessel.

Experiment 7.—Blood was taken from an arm vein and placed immediately in a small glass cup containing a few grains of powdered oxalate. The viscosity was determined at once and at the end of 10, 30, and 60 minutes.

	Viscosity.
Immediately.....	6.16
After 10 min.....	6.44
“ 30 “	6.99
“ 60 “	7.02

It is probable that stirring in the small glass cup did not give an absolutely uniform suspension of the red cells, yet the outflow time always increased in this and subsequent experiments, whereas if the change in viscosity had been due to unequal distribution of the corpuscles the results would have varied above and below the value obtained in the first determination.

Experiment 8.—10 cc. of blood were taken from an arm vein with a Record syringe containing a little powdered oxalate, placed in a separating funnel, saturated with cooled alveolar air, and stoppered. The viscosity was determined immediately and at intervals thereafter for an hour.

	Viscosity.
Immediately.....	5.48
After 10 min.....	5.48
“ 30 “	5.48
“ 60 “	5.48

1 cc. of the same blood was placed in a small glass cup and the viscosity determined immediately and at intervals for an hour.

	Viscosity.
Immediately.....	5.23
After 10 min.....	5.37
“ 30 “	5.48
“ 60 “	5.76

The difference in the initial values is attributable to an uneven distribution of the corpuscles before filling the small glass cup.

Experiment 9.—18 cc. of oxalated blood were divided into two portions, each of which was placed in a separating funnel, stoppered, and thoroughly rotated. The viscosity of blood from each funnel was taken. Funnel 1 was then left unstoppered for 4 hours and 35 minutes and the viscosity of this blood redetermined in duplicate.

	Viscosity.
Funnel 1.....	6.90
“ 2.....	6.96
“ 1 (after standing unstoppered for 4 hrs. and 35 min.)	8.25, 8.21

Experiments 7 to 9 show that the viscosity of oxalated blood constantly increases when it is allowed to stand in contact with the air, and this change can be prevented by tightly stoppering the container and rotating until carbon dioxide equilibrium is reached. The inference that the change in viscosity is dependent on the carbon dioxide content seems, therefore, justifiable.

Other observers (5) have shown that saturation of blood with carbon dioxide greatly increases the viscosity, and their findings have been confirmed in this laboratory. The influence of different partial pressures of carbon dioxide on blood viscosity is now under investigation and will be discussed in a later paper.

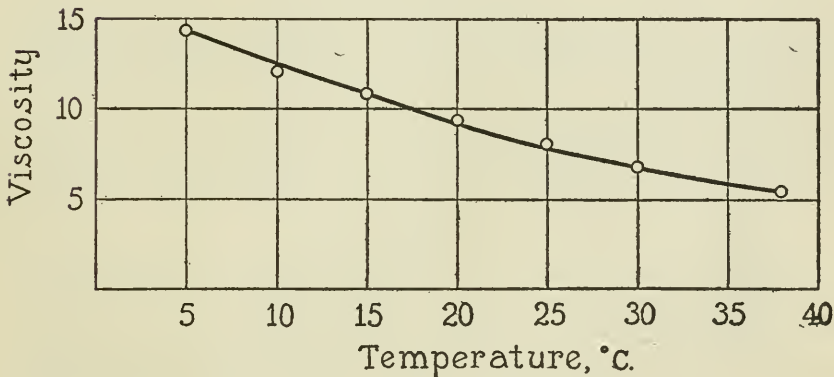
Effect of Temperature.

The blood viscosity varies greatly with temperature. It has been determined by some observers at body temperature (6) and by others at room temperature (7). Determann (8) reduced the value obtained at room temperature to 20°C. from a series of curves constructed by determining the viscosity of different bloods at different temperatures. Since all the observations in this work were made at 20°C., compari-

sons between different readings are reliable. In order, however, to study the effect of temperature on viscosity Experiment 10 was carried out.

Experiment 10.—10 cc. of blood were withdrawn from an arm vein into a Record syringe containing a little powdered oxalate, placed in a separating funnel, saturated with alveolar air, and stoppered. Blood was then withdrawn from the lower end into a small cup immediately before each determination, which was carried out in the usual manner after raising the water in the jacket to the required temperature.

Temperature. °C.	Viscosity.
5	14.41
10	12.47
15	10.96
20	9.40
25	8.03
30	6.85
38	5.48



TEXT-FIG. 1. The effect of temperature on the viscosity of blood.

When these values are plotted in the form of a curve as in Text-fig. 1, the line is almost straight. The viscosity of distilled water decreases directly with increase in temperature. With blood the viscosity is relatively slightly greater at body temperature than at lower temperature. This confirms the finding of Ewald (5).

CONCLUSIONS.

Small amounts of potassium oxalate probably have no effect on the viscosity of the blood and changes hitherto ascribed to it can be attributed either to variation in carbon dioxide content or to sedimentation of the red blood cells.

The viscosity of blood when exposed to the air increases rapidly. This change accompanies a loss of carbon dioxide and can be prevented by stoppering the container and agitating until the blood comes into carbon dioxide equilibrium with the air above it, when the viscosity remains constant.

It is essential in determining the viscosity of blood that the red cells should be uniformly suspended throughout the plasma. This can be accomplished by rotating 5 to 10 cc. of blood in a separating funnel for 1 minute.

BIBLIOGRAPHY.

1. Poiseuille, J. L. M., Recherches expérimentales sur le mouvement des liquides dans les tubes de très petits diamètres, *Ann. Chim. et Physique*, 1843, vii, series 3, 50.
2. Determann, Ein einfaches, stets gebrauchsfertiges Blutviskosimeter, *Münch. med. Woch.*, 1907, liv, 1130.
3. Burton-Opitz, R., Vergleich der Viscosität des normalen Blutes mit der des Oxalatblutes, des defibrinirten Blutes und des Blutserums bei verschiedener Temperatur, *Arch. ges. Physiol.*, 1900, lxxxii, 464.
4. Van Slyke, D. D., and Cullen, G. E., Studies of acidosis. I. The bicarbonate concentration of the blood plasma, its significance, and its determination as a measure of acidosis, *J. Biol. Chem.*, 1917, xxx, 289.
5. Ewald, C. A., Ueber die Transpiration des Blutes, *Arch. Physiol.*, 1877, 208.
6. Bence, J., Klinische Untersuchungen über die Viskosität des Blutes, *Z. klin. Med.*, 1906, lviii, 203.
7. Hirsch, C., and Beck, C., Eine Methode zur Bestimmung des inneren Reibungswiderstandes des lebenden Blutes beim Menschen, *Münch. med. Woch.*, 1900, xlvii, 1685.
8. Determann, Zur Kritik des Viskosimetrie des Blutes, *Z. klin. Med.*, 1910, lxx, 185.

BLOOD VISCOSITY.

II. EFFECT OF INCREASED VENOUS PRESSURE.

By LOVELL LANGSTROTH, M.D.

(From the Laboratories of the University of California Hospital, San Francisco.)

(Received for publication, July 22, 1919.)

Early in the study of blood viscosity the attempt was made to study the effect *in vivo* of increase of carbon dioxide on the blood viscosity by determining its coefficient before and after venous stasis induced by a loosely applied binder. The assumption was made that by raising the venous pressure for a time the passage of arterial blood through the capillaries would be delayed, the amount of carbon dioxide would be increased, and this would increase the viscosity, since it has been shown *in vitro* that saturating blood with carbon dioxide increases the internal resistance (1). It was thought that a concentration of red blood cells in the capillaries might accompany this increase in viscosity, for Cannon, Frazer, and Hooper (2) have shown that in shock the capillary count is higher than the venous.

Method.

The common method of taking blood from a finger, even when a spring lance was used, did not seem to offer the possibility of obtaining 0.2 cc. of blood rapidly enough to fill the viscosimeter without a considerable loss of carbon dioxide, and in addition it was found difficult to prevent small bubbles of air from entering the instrument. The attempt to use capillary blood for viscosity determinations was then given up and only venous blood used.

A few preliminary determinations of hemoglobin with the Palmer method (3) showed so rapid a deterioration of the standard that values obtained with it on different days were not comparable. This did not, however, affect the comparative value of determinations made on the same day, so that the 1 per cent standard was often used even though the absolute hemoglobin value was not obtained. Even

the 20 per cent solution of carbon monoxide hemoglobin as prepared in this laboratory sometimes faded rapidly in a few days. Thus a 20 per cent standard made on February 1 read 26 per cent lower when compared with a fresh one prepared on February 3. On the same day hemoglobin determinations made by the Palmer method on the same venous blood taken directly from the nozzle of the syringe with each of two 0.05 cc. pipettes, on some of the blood which had been poured into a test-tube and well agitated, and on a solution made by diluting 1 cc. of the blood to 100 cc. with 0.4 per cent ammonium hydroxide checked to within 1 per cent with the value found by the gasometric method of Van Slyke (4).

EXPERIMENTAL.

Effect of Increase in Venous Pressure on Venous and Finger Blood.

Experiment 1 shows the comparative concentration of venous and finger blood and the effect of increase in venous pressure on both.

Experiment 1.—Blood was taken with a Record syringe from an arm vein without any constriction, put into the small glass cup containing dry oxalate, and the viscosity determined in the usual manner. At the same time the finger was punctured for the red cell count and hemoglobin determination, the Palmer method being used for the latter. The blood pipettes had been carefully calibrated with mercury. A binder was then loosely applied to the arm, care being taken not to obstruct the flow of arterial blood into the arm. The determinations were then repeated after an interval of from 2 to 10 minutes. The results are shown in Table I.

TABLE I.

Venous blood		Finger blood.
Viscosity.....	6.80	
Red blood cells.....	6,490,000	6,265,000
Hemoglobin, <i>per cent</i>	133	130
Binder for 2 min.		
Viscosity.....	8.33	
Red blood cells.....	6,220,000	5,935,000
Hemoglobin, <i>per cent</i>	140	137

Experiment 2.—This experiment was similar in every respect to Experiment 1. The results are shown in Table II.

TABLE II.

Venous blood.		Finger blood.
Viscosity.....	8.3	
Red blood cells.....	4,940,000	5,520,000
Hemoglobin, <i>per cent.</i>	132	135

Binder for 2 min.		
Viscosity.....	9.40	
Red blood cells.....	5,800,000	5,980,000
Hemoglobin, <i>per cent.</i>	141	143

Experiment 3.—This experiment was similar to Experiment 1. The results are shown in Table III.

TABLE III.

Venous blood.		Finger blood.
Viscosity.....	8.69	
Red blood cells.....	6,450,000	6,250,000
Hemoglobin, <i>per cent.</i>	143	143

Binder for 2 min.		
Viscosity.....	10.6	
Red blood cells.....	5,630,000	5,300,000
Hemoglobin, <i>per cent.</i>	154	152

Except in Experiment 2 the venous count was always slightly higher than the finger count. That this is probably accidental is shown by the hemoglobin determinations which give no consistent difference between the finger and vein and check within a reasonable limit of error. It was concluded that in approximately normal individuals such as were used for this study there was no difference in the red cell concentration in the capillaries and veins that could be detected by these methods.

Effect of Increase in Venous Pressure on Hemoglobin.

After even so short a period as 2 minutes there was a very considerable rise in hemoglobin which was accompanied by a marked increase in viscosity. Experiment 4 confirms this increase in hemoglobin after venous stasis.

Experiment 4.—Blood was taken from an arm vein before and after application of a binder for 10 minutes and the oxygen-carrying power determined. From this the hemoglobin was calculated from Van Slyke's tables. The figures in Table IV show the results.

TABLE IV.

Hemoglobin, <i>per cent</i> (gasometric).....	109.3
Binder for 10 min.	
Hemoglobin, <i>per cent</i> (gasometric).....	119.1

Relation of Carbon Dioxide Content, Oxygen Unsaturation, and Oxygen-Carrying Power to Viscosity.

The attempt was made to determine whether this increase in viscosity was associated either with an increase in the carbon dioxide content or the oxygen unsaturation. Blood was taken before and after application of a binder and analyzed for total plasma carbon dioxide, plasma bicarbonate, or oxygen-carrying power and oxygen content.

Experiment 5.—Blood for the viscosity determination was taken from an arm vein with a Record syringe from which 1 cc. was immediately put into a small glass cup containing a small portion of powdered oxalate. The remainder was introduced by means of a glass tube and rubber connection into a centrifuge tube containing a little powdered oxalate and a small quantity of paraffin oil, and immediately centrifuged. The plasma was then pipetted off, the total carbon dioxide determined, and, after saturation with alveolar air, the plasma bicarbonate. Table V shows the results.

Experiment 6.—The procedure here was the same as in Experiment 5. Table VI shows the results.

Experiment 7.—This experiment differs only in the results, which are shown in Table VII.

Experiment 8.—This experiment is similar to the others. The results are shown in Table VIII.

TABLE V.

Viscosity.....	4.57
Total CO ₂ in plasma, <i>vol. per cent.</i>	57.6
Plasma bicarbonate, <i>vol. per cent.</i>	65.3

Binder for 10 min.

Viscosity.....	6.03
Total CO ₂ in plasma, <i>vol. per cent.</i>	52.8
Plasma bicarbonate, <i>vol. per cent.</i>	60.3

TABLE VI.

Viscosity.....	6.56
Total CO ₂ in plasma, <i>vol. per cent.</i>	62.7
Plasma bicarbonate, <i>vol. per cent.</i>	68.4

Binder for 10 min.

Viscosity.....	8.63
Total CO ₂ in plasma, <i>vol. per cent.</i>	63.5
Plasma bicarbonate, <i>vol. per cent.</i>	69.4

TABLE VII.

Viscosity.....	7.54
Total CO ₂ in plasma, <i>vol. per cent.</i>	59.5
Plasma bicarbonate, <i>vol. per cent.</i>	60.5

Binder for 10 min.

Viscosity.....	8.50
Total CO ₂ in plasma, <i>vol. per cent.</i>	65.3
Plasma bicarbonate, <i>vol. per cent.</i>	69.1

TABLE VIII.

Viscosity.....	8.22
Total CO ₂ in plasma, <i>vol. per cent.</i>	63.4
Plasma bicarbonate, <i>vol. per cent.</i>	65.5

Binder for 10 min.

Viscosity.....	9.73
Total CO ₂ in plasma, <i>vol. per cent.</i>	55.8
Plasma bicarbonate, <i>vol. per cent.</i>	59.6

In Experiments 5 to 8 there was a constant rather marked increase in viscosity following the rise in venous pressure. The plasma bicarbonate was constantly higher than the total carbon dioxide, but the value of neither followed consistently the viscosity, being higher in Experiments 6 and 7 but lower in Experiments 5 and 8 after increase in the venous pressure. It seemed evident from these results that the increase in viscosity following a rise in venous pressure could not be attributed to changes in the carbon dioxide content of the blood.

Experiment 9.—1 cc. of a 20 cc. sample of blood taken from an arm vein with a Record syringe was immediately placed in a small glass cup containing a very small portion of oxalate and the viscosity determined. The remainder of the specimen was passed through a glass tube and rubber connection into a test-tube containing a small amount of oxalate and a little paraffin oil and the oxygen content and oxygen-carrying power were determined. Table IX shows the results.

TABLE IX.

Viscosity.....	7.99
Venous O ₂ , vol. per cent.....	15.56
O ₂ -carrying power, vol. per cent.....	24.39
O ₂ unsaturation, vol. per cent.....	8.83
Binder for 10 min.	
Viscosity.....	8.63
Venous O ₂ , vol. per cent.....	10.99
O ₂ -carrying power, vol. per cent.....	26.73
O ₂ unsaturation, vol. per cent.....	15.74
Increase in viscosity, per cent.....	8.0
Decrease in venous O ₂ , per cent.....	29.4
Increase in O ₂ -carrying power, per cent.....	5.6
“ in O ₂ unsaturation, per cent.....	56.1

Experiment 10.—The procedure was the same as in Experiment 9. The results appear in Table X.

Experiment 11.—The procedure was similar to that in Experiment 9. Table XI shows the results.

There was a constant increase in the oxygen unsaturation values, though in Experiment 9 it was very large while in Experiment 11 it was comparatively small. The venous oxygen decreased markedly

in Experiment 9, very slightly in Experiment 10, and actually increased in Experiment 11. There was a large increase in the oxygen-carrying power in each case.

TABLE X.

Viscosity.....	6.64
Venous O ₂ , <i>vol. per cent.</i>	13.71
O ₂ -carrying power, <i>vol. per cent.</i>	24.90
O ₂ unsaturation, <i>vol. per cent.</i>	11.19
Binder for 10 min.	
Viscosity.....	7.12
Venous O ₂ , <i>vol. per cent.</i>	13.25
O ₂ -carrying power, <i>vol. per cent.</i>	30.37
O ₂ unsaturation, <i>vol. per cent.</i>	17.12
Increase in viscosity, <i>per cent.</i>	7.3
Decrease in venous O ₂ , <i>per cent.</i>	3.4
Increase in O ₂ -carrying power, <i>per cent.</i>	21.8
“ in O ₂ unsaturation, <i>per cent.</i>	29.2

TABLE XI.

Viscosity.....	6.6
Venous O ₂ , <i>vol. per cent.</i>	11.05
O ₂ -carrying power, <i>vol. per cent.</i>	24.13
O ₂ unsaturation, <i>vol. per cent.</i>	13.08
Binder for 10 min.	
Viscosity.....	7.74
Venous O ₂ , <i>vol. per cent.</i>	11.95
O ₂ -carrying power, <i>vol. per cent.</i>	26.88
O ₂ unsaturation, <i>vol. per cent.</i>	14.93
Increase in viscosity, <i>per cent.</i>	17.3
“ in venous O ₂ , <i>per cent.</i>	8.1
“ in O ₂ -carrying power, <i>per cent.</i>	11.8
“ in O ₂ unsaturation, <i>per cent.</i>	14.1

From the evidence obtained here it seemed impossible to associate the increase in viscosity with either the change in venous oxygen or the oxygen unsaturation. It ran more nearly parallel to the

rise in the oxygen-carrying power. This increase in the oxygen-carrying power could only be explained by a concentration of the blood in the capillaries; *i.e.*, by a loss of fluid from the blood to the tissues with consequent increase in the relative amount of hemoglobin. Such a concentration should be accompanied by an increase in the relative volume of the red blood cells, and experiments were therefore undertaken to determine whether this took place.

Experiment 12.—Blood was taken from an arm vein with a Record syringe and immediately placed in a small glass cup containing a very small amount of powdered oxalate. The 0.05 cc. pipette for determination of hemoglobin by the Palmer method and a capillary hematocrit tube were quickly filled from this cup. A binder was then loosely applied for 10 minutes and the determinations were repeated. The figures in Table XII show the results.

TABLE XII.

Hemoglobin, <i>per cent</i>	126.8
Hematocrit.....	41
Binder for 10 min.	
Hemoglobin, <i>per cent</i>	140.7
Hematocrit.....	54

Experiment 13.—Blood was taken from an arm vein and immediately placed in a test-tube containing a little powdered oxalate. A hematocrit tube was then filled and the remaining blood set aside for determination of the hemoglobin by the gasometric method. A binder was loosely applied for 10 minutes and the procedure repeated. The figures in Table XIII show the results.

TABLE XIII.

Hemoglobin, <i>per cent</i>	136.6
Hematocrit.....	47
Binder for 10 min.	
Hemoglobin, <i>per cent</i>	141.6
Hematocrit.....	50

These experiments showed that the increase in hemoglobin after rise in venous pressure was accompanied by a similar increase in the

relative volume of the red blood cells. In order to show *in vitro* the effect of an increase in the relative volume of the red cells on blood viscosity Experiment 14 was carried out.

Experiment 14.—16 cc. of blood were taken from an arm vein with a Record syringe containing a little powdered oxalate, 8 cc. being placed into each of two tubes and a few drops of paraffin oil added. After allowing the corpuscles to settle 0.8 cc. of plasma was removed from one of the tubes and both were then thoroughly stirred under oil by up and down movement of a stirring rod flattened at the lower end to nearly the inside diameter of the tube. 1 cc. of whole blood was removed from each tube and the viscosity determined.

	Viscosity.
Whole blood.....	9.59
Blood minus 10 per cent of volume in plasma	12.33

This experiment demonstrated that an increase in the relative volume of the red blood cells caused an increase in the viscosity of the whole blood.

It was of further interest to discover whether the fluid lost to the tissues was plasma or whether the plasma itself became more concentrated by loss of fluid and therefore more viscous. Experiment 15 was undertaken to determine this point.

Experiment 15.—15 cc. of blood were taken from an arm vein with a Record syringe containing a little dry powdered oxalate, and put into a centrifuge tube. A specimen was removed for determination of the oxygen-carrying power, a few drops of paraffin oil were added, and the remainder was centrifuged at high speed so that the plasma could be pipetted off. The total nitrogen of 1 cc. of plasma and the plasma viscosity were determined. A binder was loosely applied to the arm for 10 minutes and the procedure repeated. The figures in Table XIV show the results.

TABLE XIV.

O ₂ -carrying power, vol. per cent.....	24.3
Viscosity of plasma.....	1.89
Total nitrogen in 1 cc. of plasma, gm.....	0.0105
Binder for 10 min.	
O ₂ -carrying power, vol. per cent.....	27.9
Viscosity of plasma.....	2.30
Total nitrogen in 1 cc. of plasma, gm.....	0.0126

This experiment showed that a rise in venous pressure was accompanied by an increase in viscosity and total nitrogen of the blood plasma, and therefore by a concentration of the plasma. An increase in the viscosity of the plasma is therefore a factor in the increase in the viscosity of the whole blood after a rise in venous pressure.

CONCLUSIONS.

A rise in venous pressure caused by application of a loose binder to the arm results in a marked increase in the viscosity of the whole blood which is primarily due to a concentration of the blood in the capillaries. This concentration is shown by an increase in the viscosity and total nitrogen of the plasma, an increase in the relative volume of the red blood cells, and an increase in the relative percentage of hemoglobin.

Change in the viscosity of whole blood following venous stasis apparently bears no demonstrable relation to the carbon dioxide or oxygen content.

BIBLIOGRAPHY.

1. Bence, J., Klinische Untersuchungen über die Viskosität des Blutes, *Z. klin. Med.*, 1906, lviii, 203.
2. Cannon, W. B., Frazer, J., and Hooper, A. N., Some alterations in distribution and character of blood in shock and hemorrhage, *J. Am. Med. Assn.*, 1918, lxx, 526.
3. Palmer, W. W., The colorimetric determination of hemoglobin, *J. Biol. Chem.*, 1918, xxxiii, 119.
4. Van Slyke, D. D., Gasometric determination of the oxygen and hemoglobin of blood, *J. Biol. Chem.*, 1918, xxxiii, 127.

THE EFFECT OF HYPOTONIC AND HYPERTONIC SOLUTIONS ON FIBROBLASTS OF THE EMBRYONIC CHICK HEART IN VITRO.

BY MARY JANE HOGUE, PH. D.

(*From the Department of Embryology of Carnegie Institution of Washington, Baltimore, Md.*)

PLATE 56,

(Received for publication, August 19, 1919.)

In the literature on tissue culture are reported, more or less completely, experiments testing the effect of hypotonic and hypertonic solutions upon growth of cells and upon individual cell structures. It was with a view, therefore, of studying more fully the effect of solutions of different concentrations that the present work was undertaken. The experiments were performed in April, May, June, and July, when the weather was at summer heat and there was consequently little danger of chilling the delicate new growths, which are susceptible to any sudden change of temperature. This point is important, because several investigators have stated definitely that growth is much better in the spring and summer than in the fall and winter.

Carrel and Burrows were the first to try the effect of dilution and concentration of the medium upon tissue cultures, using the spleen of adult chicks and of 14 and 16 day chick embryos. Distilled water was added to the plasma to make it hypotonic, and sodium chloride to make it hypertonic. They found that growth in hypertonic plasma was always less than normal, while growth in the hypotonic solution (three volumes of plasma to two volumes of distilled water) was greatly accelerated. In fact, this medium gave the greatest amount of growth, much more than that obtained in normal plasma, a medium which these writers admit is not the best for this purpose.

Ruth found that by adding distilled water to plasma in which epithelial cells of frog skin were planted the growth was greatly increased.

Later, Lambert tried diluting the plasma with serum. He got the most extensive migration in plasma diluted with two parts of serum. Dilution with serum, Ringer's solution, and water gave about the same results. He therefore

concluded that "the most active migration took place in the plasma diluted with the largest quantity of fluid, and not in the medium made hypotonic by the addition of distilled water." This agrees with the work of Burrows (1913), who used spleen and diluted the plasma with serum. Both these observers attribute the increased migration to a diminution in the amount of fibrin in the clots; this makes a coarser meshwork which offers less resistance to the progressive movement of the cells. Lambert also finds that diluting the plasma affects differently the various types of cells. Actively motile cells, such as those of bone marrow and spleen, show increased migration; cells with limited power of locomotion, like those of intestinal epithelium, are not affected by the dilution of the medium; while cells of moderate motility, such as connective tissue cells, are only slightly influenced by it. Lambert concludes that this dilution of the medium has likewise no effect upon cell division.

Ebeling used hypotonic, hypertonic, and diluted media prepared by adding Ringer's solution to normal chick plasma and embryonic chick extract. Each of these media at first stimulated cell proliferation, but after a few days this decreased and ended in death unless measures were taken to revive the cultures.

Sundwall also grew tissues in hypotonic solutions, using plasma diluted with distilled water. He found the growth to be more prolific, but the difference between this and growth in plasma was not so marked as that obtained by Carrel and Burrows.

Cash used hypotonic salt solution on 3 day chick cultures and found that at once numerous vesicles appear, change shape characteristically for a short time, and then flow back into the cells. He states that it is difficult to produce these changes in younger cells; *i.e.*, in growths 1 to 2 days old.

Goldschmidt found that the direction of growth of the sex cells of the moth (*Samia cecropia* L.) was affected by hypertonic and hypotonic solutions. In the former the cells grew out against the follicle wall. The extent of outgrowth depended upon two factors—the age of the cell and the hypertonicity of the medium. The whole process was reversible without change of medium, as were also the slight changes produced by hypotonic media. Goldschmidt therefore concludes that the follicle membrane has active control of osmotic conditions inside the follicle.

Lewis and Lewis (1914-15) note a definite effect of hypotonic and hypertonic solutions upon the mitochondria; in the first they swell, while in the second they shrink. Cowdry states that very dilute solutions of formaldehyde cause swelling of the mitochondria, while a concentrated solution results in shrinkage.

Material.

For these experiments hearts of 6, 7, 8, and 9 day chick embryos were used. The medium was a modification of Locke's, known as Locke-Lewis solution. The Locke formula was as follows:

	gm.
Sodium chloride.....	0.9
Calcium chloride.....	0.024
Potassium chloride.....	0.042
Sodium bicarbonate.....	0.02

This was modified, according to Lewis' method (1915), by the addition of chick bouillon (15 cc. of bouillon and 0.25 gm. of dextrose to 85 cc. of Locke's solution), sterilized in clean test-tubes in an Arnold sterilizer, and kept in a moist chamber to avoid evaporation. In order to make it hypertonic the solution was boiled down one-fourth, one-half, etc., as required, and the amount of sodium chloride present was calculated. The hypotonic solutions were made by diluting the Locke-Lewis solution with distilled water, and in these also the amount of sodium chloride was determined. Since chick bouillon contains practically the same amount of sodium chloride as the Locke solution, the calculation of this content in the Locke-Lewis solution was made upon the same basis. It will be noted that only the amount of sodium chloride has been considered, although the other salts were present in definite amounts and always in the same proportion.

Method.

All the instruments and glassware used in the experiments were sterilized; the instruments were sterilized in a flame continually during the preparation of the cultures, so that infection of the cultures was rare. The solution was warmed and poured into small Petri dishes. The heart was aseptically removed from the chick embryo in Locke-Lewis solution and transferred to another Petri dish containing about 10 cc. of the medium. Here it was cut into small pieces and transferred by means of a pipette to a cover-glass, which was immediately inverted over a hard vaseline ring on a depression slide. The chance of evaporation by this method of the Lewises is very small, as the tissue can be placed on the cover-glass and sealed in a few seconds. When the tissue was to be planted in hypertonic or hypotonic solution the heart was placed in the liquid immediately after its removal from the embryo; the tissue was therefore never in contact with other solutions that might possibly dilute the medium.

The cultures were incubated in a constant temperature box at 39°C. Many of the slides were treated with neutral red which stains certain granules and vacuoles (Lewis, 1919). Janus black No. 2 was added when the mitochondria were to be studied, but only when early results were obtained from the experiment, as the cells do not live much more than an hour after being stained with it.

Normal Growth of Heart Tissue.

The normal growth of heart tissue is characteristic. Two types of connective tissue cells appear—fibroblasts and mesothelial cells. The fibroblasts (Fig. 1, *a*) are of more frequent occurrence and show two methods of growth: (1) migratory (Fig. 1), where the cells are scattered along the transplant and out into the medium; and (2) reticular, where the cells form a close reticular network. The latter is usually the more vigorous growth. These cells have long, branching processes, often so thin that it is impossible to follow them in their farthest wanderings and anastomoses with other cells. Hence, in most of the drawings of cells these processes are omitted, as they could not be seen with the camera lucida.

The mesothelial cells (Fig. 1, *b*) are of less frequent occurrence. They always appear in membrane formation (Lewis and Lewis, 1912), sometimes as small inclusions among the fibroblasts, and again as large growths completely surrounding the transplant. They are much larger and hardier, and withstood the action of the hypotonic and hypertonic solutions much better than the sensitive fibroblasts, with their fine, branching processes, which usually reacted at once to the new medium. For that reason, and also because they were the more common form, the fibroblasts were always used in the experiments.

As controls to the other experiments 282 cultures were planted in normal Locke-Lewis solution, and of these 188 (66.6 per cent) grew. The length of life in this medium varied. The oldest growth was 19 days old, and had been made from a 7 day chick embryo. It lived to this age without any change of the medium; in fact it was sealed with a vaseline ring during the entire time. The cells contained very few fat globules and towards the end of the experiment they withdrew their processes, only blunt ends remaining. They then accumulated in groups of five or six all over the slide.

Experiments with Hypotonic Solutions.

For these experiments three different solutions were employed: (1) three volumes of Locke-Lewis solution to two volumes of distilled water; this had a sodium chloride content of 0.540 per cent; (2) one volume of Locke-Lewis solution to one of distilled water; sodium chloride content 0.45 per cent; and (3) one volume of Locke-Lewis solution to three volumes of distilled water; sodium chloride content 0.225 per cent.

The first solution, which was the proportion used by Carrel and Burrows in their work on the spleen, was employed in two experiments with hearts of chick embryos 8 and 9 days old respectively. Out of twenty-three cultures seven gave reticular growth, thirteen migration, and three showed no signs of growth. In one experiment all were dead on the 4th day, while in the other some of the cultures lived for 7 days. Migration of cells was at first rapid as compared with the controls, but the latter lived a little longer. The percentage of growths in this medium is high (86.9), but only two experiments were performed. On these 2 days the percentage of growth of cultures in normal Locke-Lewis solution was 81.8—only slightly less. The number of reticular growths in the two media was practically the same—35 per cent in hypotonic and 30 per cent in Locke-Lewis solution, although in the former the extent of growth was greater.

Burrows (1913) and Lambert attribute the increased migration to a lessening of fibrin in the diluted plasma and consequent decrease in the resistance offered to migration. Here, however, this was not the case, as there was no fibrin in any of the cultures grown in the Locke-Lewis solution. The real cause seems to me to be the search for food which acts as a stimulus, as will be seen later. Burrows (1913) has also observed that tissues planted in plasma diluted with water suffer early death. This he thinks is due to the hypotonicity. He says: "They die apparently in the plasma diluted with salt solutions from an early exhaustion of food materials." This I believe is true, although when they die after being treated with (not grown in) hypotonic solution, it is possible that the cells have taken up too much water, which of itself is known to act as a poison,

causing death. When the individual cells were examined, a few were found to be long and fusiform and often small, though many were in a vigorous condition. When neutral red and Janus black No. 2 were added to the drop of culture fluid, both stains being made up in the hypotonic fluid used for the experiment, the neutral red granules were found to be arranged around the centriole near the nucleus, just as in the cells grown in Locke-Lewis solution. The mitochondria were long, sometimes branching, and often formed a network. Sometimes they extended down the processes to a considerable length. All these forms are as one usually finds them in normal growths.

The most striking point about the growths in this solution, and indeed in all hypotonic solutions, was that the cells nearest the explant died first (Fig. 2). This is exactly the reverse of the condition prevailing in all Locke-Lewis cultures and in the hypertonic growths, where the cells at the outer edge of the growth die first, until finally only those next to the transplanted tissue are left. These frequently live on for days after the main part of the growth has died. In the hypertonic solutions the migration is slower. There is a greater amount of food material (salts, etc., from the Locke solution and chicken bouillon) in the medium, so that the cells do not need to migrate so rapidly and consequently do not give off so much in the form of waste products. Hence the medium is not altered so rapidly (Burrows, 1913) and migration is retarded. In short, all depends upon the amount of food in the medium. When this is small, as in hypotonic solutions, the cells must migrate more rapidly to obtain food. They then give off more waste products which alter the medium, thus further stimulating movement. In a short time the accumulation of waste products becomes so great and the supply of food near the explant so small that the cells in that vicinity die. The same phenomenon was observed in *Amæba limax* cultures grown on agar (Hogue). The amebæ nearest the old point of inoculation on the Petri dish die before those which have wandered out over the dish, where they are free from waste products and can get more food and oxygen.

In the second hypotonic solution, made with one part of Locke-Lewis solution to one part of distilled water, 90 out of 202 explants grew (44.5 per cent). The migration was not so rapid as in the former

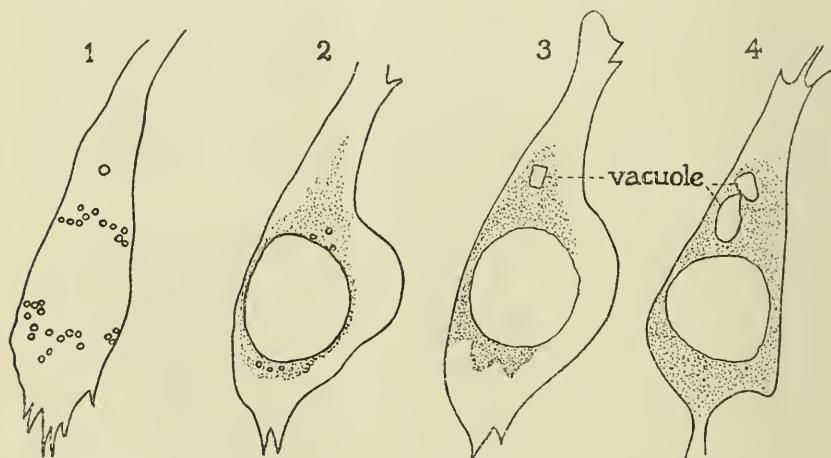
medium (0.54 per cent sodium chloride), and consequently the cells survived longer. On one slide they lived for 12 days without a change of the solution. As a rule, the cells appeared normal, with large growths in both reticular and migratory formation. The individual cells were normal in shape and the neutral red granules were usually the same as those in tissue grown in Locke-Lewis solution. Even the mitochondria presented a normal appearance. The cells seemed to have completely adapted themselves to their hypotonic environment. In this medium, as in the former one, the first cells to die were those next to the tissue explant.

The third hypotonic solution (one part of Locke-Lewis solution to three parts of distilled water, with a sodium chloride content of 0.225 per cent) gave no growth whatever after 48 hours of incubation. One drop of Locke-Lewis solution was added to some of the slides and the tissue again incubated in the hope of inducing migration, but the cells appeared to be dead.

Hypotonic Plus Hypotonic Solution.—The fact that excellent growths had been obtained on some slides of the Locke-Lewis 0.45 per cent sodium chloride medium while there was none on others planted the same day, in the same medium, and from the same heart, suggested the possibility that the particular explants that grew so well may have contained sufficient plasma of their own to change the medium. Accordingly, to the 24 and 48 hour growths in this medium was added some of the same fresh solution. On many slides the cells were all dead on the following day. On others the cells at the edge of the growth, which were very flat and thin with a large surface exposed, had been killed by the new hypotonic solution, while others had migrated out over them and were still in good condition (Fig. 3), showing that they retained the power of adapting themselves to the medium. It is not possible to determine here whether the explant had taken sufficient plasma with it to modify the medium, or whether the resistance of the individual cells was greater on some slides than on others. This individual difference in the cells is a factor always to be considered. It makes experimenting with tissue cultures a difficult problem, and one must perform a large number of experiments in order to draw any trustworthy conclusions.

Effect of Hypotonic Solutions on Normal Growth.

Hypotonic Solution, 0.225 Per Cent Sodium Chloride.—When a normal growth of 48 hours was treated with hypotonic Locke-Lewis solution with 0.225 per cent sodium chloride content, it was killed almost at once. The cells could therefore be stained with Janus black No. 2 without danger of interfering with the results of the experiment. After the growths had been stained with neutral red and Janus black, drawings were made of certain cells and the hypotonic solution was added.



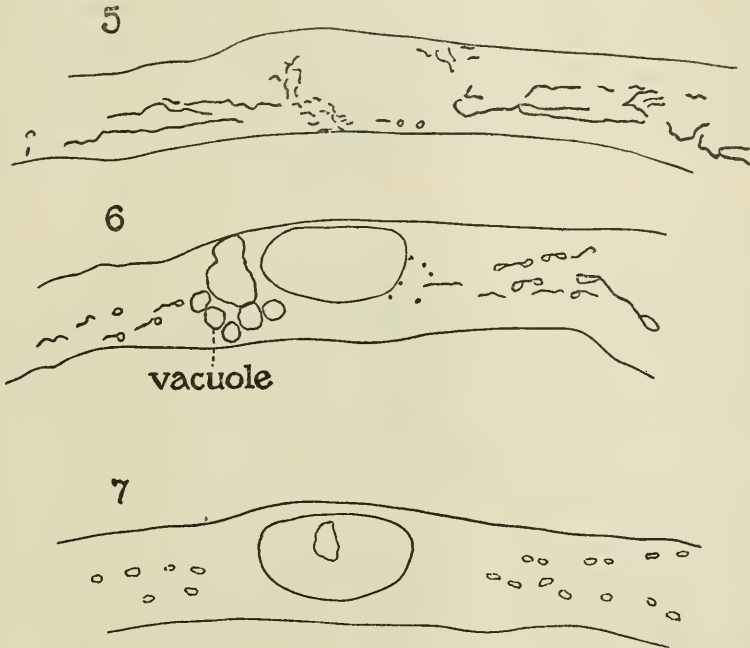
All the text-figures are camera lucida drawings made from living cells which have been stained with neutral red and often also with Janus black No. 2. The neutral red granules are represented as small circles, the mitochondria as solid lines or solid rods.

TEXT-FIG. 1. A normal fibroblast from a 48 hour growth explanted from a 9 day chick embryo heart and stained to show the neutral red granules.

TEXT-FIGS. 2 to 4. The same cell after treatment with Locke-Lewis solution (0.225 per cent sodium chloride content). Text-fig. 2 shows the cytoplasm much enlarged by the intake of water and divided into a granular and a clear area. The nucleus is swollen and has a distinct nuclear wall. A few neutral red granules are still visible. In Text-figs. 3 and 4 vacuoles are appearing. In Text-fig. 4 the cell is becoming smaller. Death changes have set in.

Many of the cells immediately took up a large amount of water. Text-fig. 1 shows a cell stained with neutral red, in which the gran-

ules are grouped together around the centriole. Text-fig. 2 represents the same cell 8 minutes after treatment with the Locke-Lewis sodium chloride 0.225 per cent solution. Here we have two kinds of cytoplasm, a granular part around the much swollen nucleus, and a clear outer area which has taken up water in the process of equalizing the osmotic pressure within the cell incident to the new environ-



TEXT-FIG. 5. Normal fibroblast from a 48 hour growth explanted from a 9 day chick embryo heart.

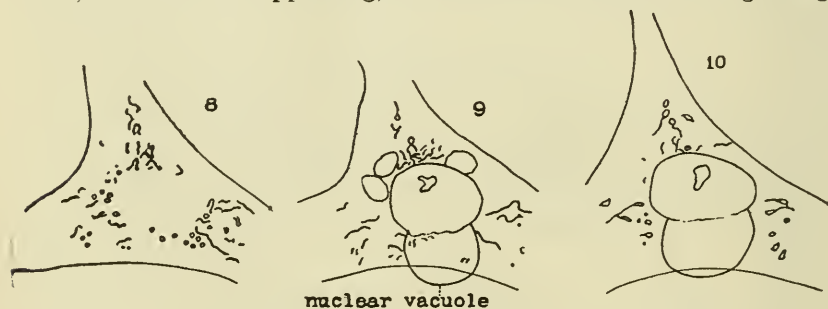
TEXT-FIG. 6. The same cell 5 minutes after treatment with Locke-Lewis solution (0.225 per cent sodium chloride content). The mitochondria are forming vesicles at their ends. Vacuoles are appearing in the cytoplasm.

TEXT-FIG. 7. The same cell 55 minutes after treatment. Only faintly staining vesicles remain where the mitochondria were.

ment. The processes have become more distinct as they, too, are full of the absorbed water. In Text-fig. 3 the neutral red color has disappeared from the granules and the nuclear wall is very distinct—both indications of cell death. In Text-figs. 3 and 4 vacuoles are appearing. Text-fig. 4 was drawn 4 hours, 25 minutes after the cell

was treated. Again the cell is small and most of the clear cytoplasm has been filled up with fine granules. This slide was later stained with neutral red, but the new vacuoles did not take up the stain, having undergone some death changes.

When the reaction of the cell to the hypotonic solution was not too sudden the effect on the mitochondria could be followed. Text-fig. 5 shows a cell with long branching mitochondria, drawn at 3.17 p.m. It was treated with hypotonic Locke-Lewis 0.225 per cent sodium chloride solution at 3.20 p.m.; by 3.25 p.m. (Text-fig. 6) no neutral red granules could be seen, the nucleus had a distinct membrane, vacuoles were appearing, and the mitochondria were beginning



TEXT-FIG. 8. Normal fibroblast from a 9 day chick embryo heart; 48 hours growth.

TEXT-FIGS. 9 and 10. The same cell 30 and 50 minutes after treatment with Locke-Lewis solution (0.225 per cent sodium chloride content). All the neutral red granules have disappeared. The mitochondria are forming vesicles. Vacuoles have appeared in the cytoplasm and a nuclear vacuole has formed.

to degenerate. In some, either one extremity or both seemed to swell (Text-fig. 6). Gradually the connection between these two small vesicles became fainter, then disappeared, and finally there remained only a faintly staining pale blue vesicle of irregular outline where a large mitochondrion had been (Text-fig. 7). The small mitochondria had disappeared entirely.

This process is also seen in Text-fig. 8 (normal), and in Text-figs. 9 and 10, drawn respectively 30 and 50 minutes after treatment.

Not all the mitochondria in a cell form these vesicles while undergoing degeneration, nor do those which do form them appear to bear any relation to the nucleus. Some are near it, some are farther away

near the cytoplasmic processes. In no case did I observe a swelling of the mitochondria as Lewis and Lewis (1914-15) and Cowdry claim occurs in hypotonic solutions. In every experiment the neutral red color disappeared from the granules almost as soon as the hypotonic solution was added. The mitochondria are much slower in disappearing.

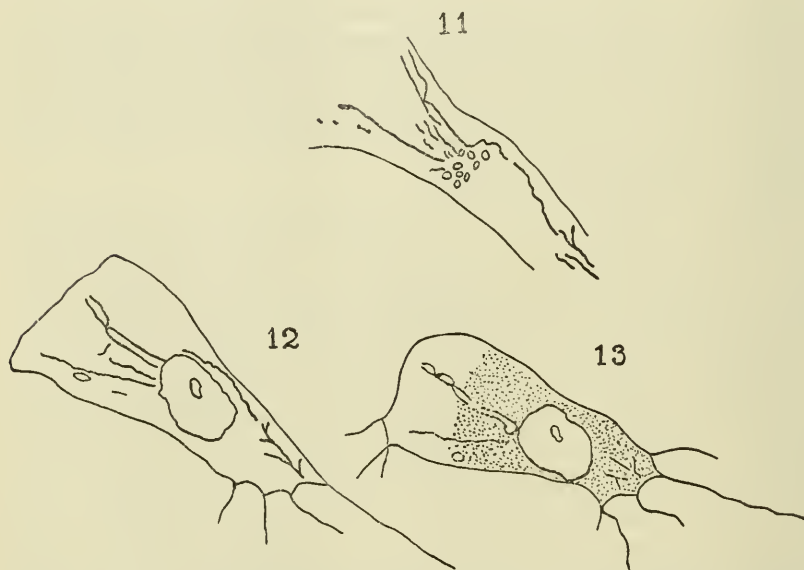
Hypotonic Solution, 0.3 Per Cent Sodium Chloride.—When the normal cells were treated with hypotonic Locke-Lewis solution with 0.3 per cent sodium chloride content, practically the same results as those recorded above were obtained. Certain mitochondria were followed and most of them simply faded from view. A few first formed a small vesicle at one end.

Hypotonic Solution, 0.45 Per Cent Sodium Chloride.—Hypotonic Locke-Lewis solution with 0.45 per cent sodium chloride content was tried on normal 48 hour growths. One cell (Text-fig. 11) with large, characteristic mitochondria was followed closely for 6 hours. Two large mitochondria in the anterior end were crossed; near them was a long, slender mitochondrion extending almost to the nucleus, and in the posterior end were some shorter ones with characteristic shapes. When treated with hypotonic solution the cell reacted at once. The color in the neutral red granules soon disappeared and the nuclear wall became distinct and irregular in outline. The mitochondria did not change shape at first but gradually became fainter (Text-fig. 12). The two peripheral ends of the long crossed mitochondria seemed to come together to form a ring (Text-fig. 13). About half way down the mitochondria there was a break and the parts nearer the nucleus became granular. About 1 p.m. it was noticed that these granular parts of the mitochondria seemed to be connected with a small clear vesicle near the nucleus. It looked as though they were forming a tubule which opened into the vesicle.

During the next 3 hours there was little change in the mitochondria, except that they grew more slender and faint. The cytoplasm around them became more granular, so that it was difficult to follow them. At 4.15 p.m. they were finely granular; this condition persisted for 3 days, when the slide was destroyed. The long mitochondrion to the right of those which were crossed did not appear so granular. It seemed, like the distal ends of those which were crossed,

to become more slender as though some substance were dissolving away from it. I could not observe, however, that any of the mitochondria became shorter.

In some of the cells treated with hypotonic Locke-Lewis solution large, clear vacuoles forming around the nucleus were frequently observed (Text-figs. 9 and 10). Others would form in the cytoplasm,



TEXT-FIG. 11. Normal fibroblast from a 9 day chick embryo heart, 48 hours growth, showing characteristic mitochondria and neutral red granules grouped around the centriole area.

TEXT-FIG. 12. The same cell 2 hours later. The neutral red granules have disappeared. The nuclear wall is distinct and the mitochondria are becoming fainter.

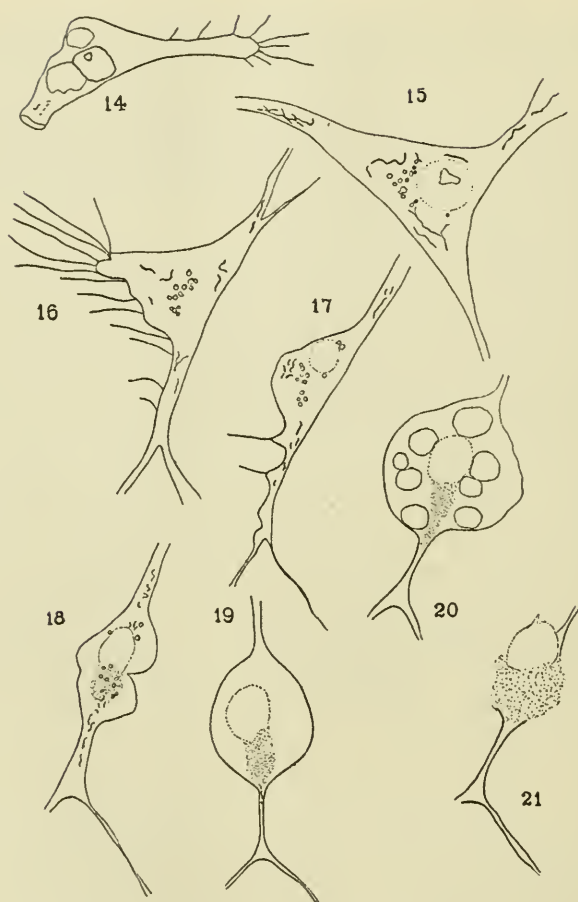
TEXT-FIG. 13. The same cell 2 hours later. The cytoplasm is granular. The two long crossed mitochondria are broken into pieces, some of which are granular. The other mitochondria are fainter or have disappeared entirely.

but these did not have the regular round or oval outlines of the nuclear vacuoles where the surface tension seemed to be very great. The nucleus, which was usually swollen in these cases, had taken up more water than it could hold and had broken out to form a vacuole. The nuclear wall near the vacuole was irregular in outline.

As already stated, the cells frequently become swollen with the intake of water as soon as they have been treated with the hypotonic solution. They remain in this condition for an hour or so, until the cell has begun to undergo the changes following death, when they show shrinkage. At times all the processes at one end are withdrawn and the end of the cell rolls back on the cell itself, as though from sudden contraction (Text-fig. 14).

A few experiments were tried with tissues which had failed to grow in Locke-Lewis 0.45 per cent sodium chloride solution. To each was added a drop of normal Locke-Lewis solution and the slides were again incubated, but no migration of cells ever appeared. A similar experiment was tried with tissue that would not grow in normal Locke-Lewis solution. Distilled water was added to the slides but the tissues did not respond, even after being in the incubator 48 hours. They appeared to have lost the power to migrate. They were not dead, for the pieces of heart frequently continued to contract, although cell migration could not be induced.

It was found that when hypotonic Locke-Lewis 0.45 per cent sodium chloride solution was added to normal growths it did not always kill the cells. A series of general experiments with this solution was tried with 24 and 48 hour growths. After thoroughly washing the cultures with the solution they were put in the constant temperature chamber at 39°C. and observed from hour to hour in a warm box. In a short time they took on a very different appearance from the normal. The cells were spider-like, their form completely changing from the flat, triangular, and fusiform shape, with long, fine processes almost invisible at the ends. Now the processes had a definite outline and seemed to anastomose, and the cell bodies had thickened into round or oval forms. Sometimes only the cells at one side of the explant would be thus affected, at other times those at one end, and again, all the cells. After 24 hours some of these cells would be dead, others would have recovered, and growth and migration would be going on beyond the dead cells. In one series of experiments pieces from the same heart were planted in hypotonic Locke-Lewis 0.45 per cent sodium chloride solution and in normal Locke-Lewis solution. Growth took place in both. When the controls were 24 and 48 hours old they were treated with hypotonic



TEXT-FIG. 14. Fibroblast half an hour after treatment with Locke-Lewis solution (0.45 per cent sodium chloride content), showing one end rolled over onto the cell as if from contraction.

TEXT-FIG. 15. Normal fibroblast from a 7 day chick embryo heart; 4 days growth.

TEXT-FIGS. 16 to 21. The same cell after treatment with Locke-Lewis solution (1.8 per cent sodium chloride content). Text-fig. 16 shows long fine processes and a condensation of the cytoplasm. In Text-figs. 17 and 18 the processes have been withdrawn and blebs are forming. The mitochondria are shorter and more condensed. In Text-fig. 19, a large bleb surrounds the nucleus and granular cytoplasm. Text-figs. 20 and 21 show death processes. Vacuoles appear and the cell wall breaks down leaving only the nucleus and granular cytoplasm.

Locke-Lewis 0.45 per cent sodium chloride solution. Some were killed while others grew.

The effects following the addition of the hypotonic 0.45 per cent sodium chloride solution were varied. On some slides all the cells were killed; on others only a few, and migration went on beyond them; on still others the solution acted as a stimulant and growth and migration were greatly accelerated. Here again the condition of the cells in the original growth must have had a great influence on the results. While I tried to take only slides which had good reticular growths, there must have been great individual differences, as even in normal Locke-Lewis solution some cultures will live much longer than others made from the same heart, in the same medium, and at the same time.

Summary of Results with Hypotonic Solutions.

1. Locke-Lewis solutions were made hypotonic by the addition of distilled water. They were used with a sodium chloride content of 0.54, 0.45, 0.3, and 0.225 per cent respectively.

2. Tissue grew in the first two solutions, which acted as a stimulus.

3. The tissues did not live so long in these media as in normal Locke-Lewis solution, but growth was more rapid.

4. The cells of normal growth were killed by treatment with hypotonic solutions with a sodium chloride content of 0.3 and 0.225 per cent respectively.

5. The cells absorbed much water, as did also the nucleus, which frequently formed a nuclear vacuole as an outlet for the extra amount of liquid absorbed.

6. Neutral red vacuoles and granules soon lost their color when the cells were treated with the hypotonic solutions that caused their death.

7. Mitochondria were not affected by the hypotonic solutions, but as the cell died vesicles formed at the extremities and persisted after the rest of the mitochondrion had disappeared; or the mitochondria broke up into granules or simply became more slender until only a faint, rough outline was visible.

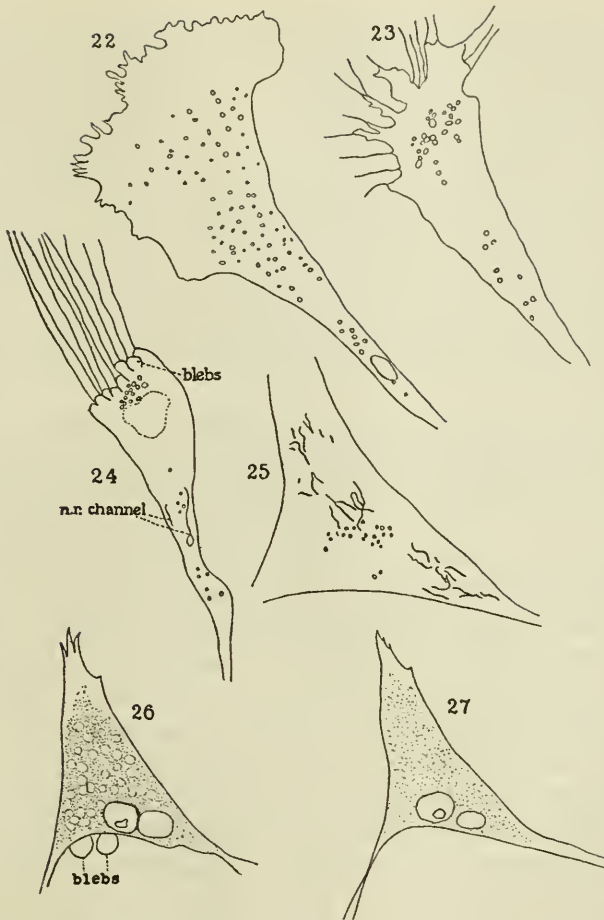
Experiments with Hypertonic Solutions.

Three grades of hypertonic Locke-Lewis solution, containing respectively 1.8, 1.5, and 1.2 per cent of sodium chloride, were used in this series of experiments.

Hypertonic Solution, 1.8 Per Cent Sodium Chloride.—Thirty-eight transplants were made, not one of which showed any signs of growth. It was evident that the fluid was too hypertonic to induce cell migration. Next the effect of this medium was tried upon tissue that had been growing well in normal Locke-Lewis solution for 2, 3, and 4 days respectively. In all the cells were killed within 3 or 4 hours, and in some within 1 hour after treatment.

The effect was followed on certain individual cells which were first stained with neutral red and Janus black No. 2. Text-fig. 15 is a cell taken from a 4 day growth of a 7 day chick heart after it had been treated with neutral red and Janus black at 11.15 a.m. At 11.20 the Locke-Lewis 1.8 per cent sodium chloride solution was added. By 11.25 the cell had begun to contract and crumple up like folds in a piece of cloth. At 11.30 (Text-fig. 16) the body of the cell was thick, and from one side and from the ends, where there had been processes, fine, thread-like structures extended out into the medium. By 11.40 (Text-fig. 17) most of these processes had been withdrawn; by 12.02 p.m. (Text-fig. 18) all the side processes had been withdrawn and the central granular part was surrounded by a clear, flat area. In Text-figs. 16 and 17 both mitochondria and neutral red granules are arranged as in normal growth. In Text-fig. 18 the mitochondria have become shorter and more concentrated in the center of the cell. At 12.15 (Text-fig. 19) the neutral red granules and mitochondria had disappeared and the cell was dead. Text-figs. 20 and 21, drawn at 12.20 and 12.40, show further death changes. In Text-fig. 20 large vacuoles are appearing in the clear areas. These later break down, leaving only the granular cytoplasm and nucleus (Text-fig. 21).

Text-figs. 22 to 24 show the contraction of the cytoplasm and the concentration of the neutral red granules at the end where the nucleus is located. The fine processes at the large end were invisible at 10.07 a.m. when the cell was treated with Locke-Lewis 1.8 per cent



TEXT-FIGS. 22 to 24. Cell from a 4 day growth of a 7 day chick embryo heart, stained with neutral red and treated with Locke-Lewis solution (1.8 per cent sodium chloride content). The processes at the anterior end have become visible in Text-fig. 22. Text-fig. 23 shows a contraction of the cytoplasm and a partial withdrawal of the anterior end to form thread-like processes. These processes are much longer in Text-fig. 24 where neutral red channels are shown opening into neutral red vacuoles.

TEXT-FIG. 25. Fibroblast from a 7 day chick heart; 4 days growth.

TEXT-FIGS. 26 and 27. The same cell after treatment with Locke-Lewis solution (1.8 per cent sodium chloride content). The alveolar structure of protoplasm is shown (Text-fig. 26), which 1 hour later (Text-fig. 27) became finely granular.

sodium chloride solution. By 10.10 (Text-fig. 22) they had contracted until they were plainly visible. Neutral red vacuoles are scattered over the cell, a very large one being in the posterior long process, only part of which was drawn. By 11.45 (Text-fig. 23) the cell had shrunk appreciably, the neutral red granules were concentrated at the anterior end, and thin processes were forming. At 12.35 p.m. (Text-fig. 24) these looked like long streamers. It appeared as though the anterior end of the cell had been attached at places to the cover-glass, and that when the cell began to shrink from loss of water the cytoplasm was pulled out into these long processes. When the pull was too great they lost hold and were rapidly withdrawn into the cell.

Coincident with the formation of these protoplasmic streamers, blebs—clear, round cytoplasmic protrusions—appeared behind them. Toward the center of the cell neutral red channels opened into the large neutral red vacuole. In many of the cells these channels were branching and anastomosing. This particular cell was dead at 1.45.

To those who still believe in the alveolar and reticular structure of protoplasm Text-figs. 25 and 26 will prove of interest. Text-fig. 25 shows a cell stained with Janus black and neutral red which bring out the mitochondria and the neutral red granules. At 11.20 a.m. the tissue was treated with Locke-Lewis 1.8 per cent sodium chloride solution. This cell did not contract so much as many of the others, nor were there formed the long, fine processes which I have found so characteristic of death in these hypertonic solutions. The cytoplasm, however, became alveolar (Text-fig. 26), small, round spaces appearing over the granular part of the cell by 12.25 p.m. The nuclear wall was very distinct, the granules no longer showed the neutral red color, and the mitochondria had disappeared—all evidences of cell death. By 1.45 (Text-fig. 27) these alveoli had also disappeared and the protoplasm had become granular. It would seem quite certain, therefore, that the alveolar structure described by the early cytologists was an artifact caused by the fixing solution, which was hypertonic to the protoplasm.

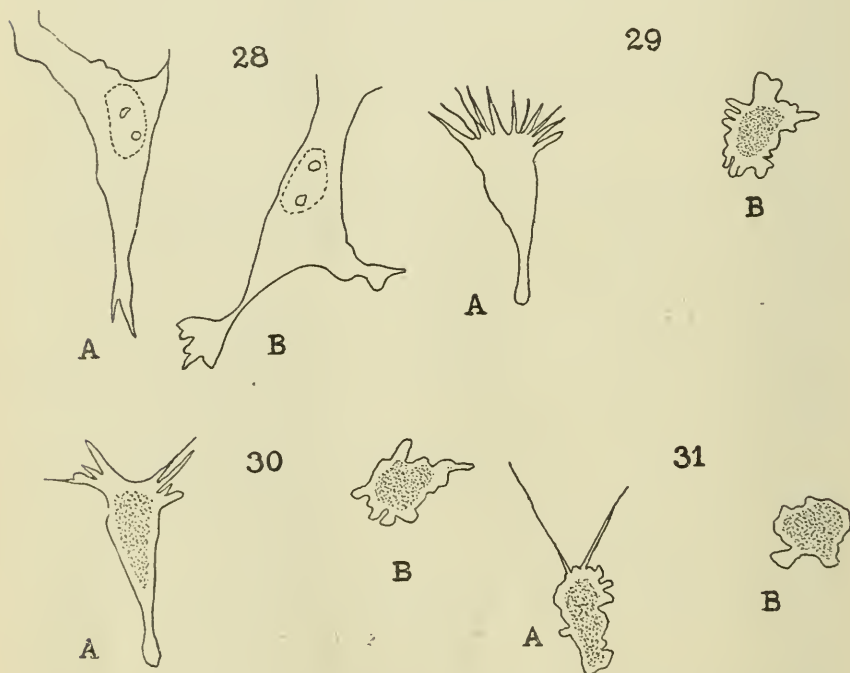
Hypertonic Solution, 1.5 Per Cent Sodium Chloride.—In spite of the fact that several authors have stated that tissues will not grow at all, or, at best, very poorly in hypertonic solutions, I have neverthe-

less grown a few in hypertonic Locke-Lewis solution with a sodium chloride content of 1.5 per cent. Out of 130 explants of heart tissue in this medium, twelve (9 per cent) showed migration and reticular formation. One of these lived 7 days. The migration was not great, and only in a few instances were there reticular growths.

I was interested in learning how the mitochondria would be affected by this new medium stained with Janus black No. 2 and neutral red. The mitochondria in many of the slides were long, irregular, and spread out normally into the processes of the cells. The neutral red granules were also arranged around the centriole and the mitochondria mingled with them as in normal cells. Occasionally the mitochondria were round or slightly oblong, but this was undoubtedly due more to the generally poor condition of the growth than to the hypertonic medium. The same round mitochondria are found on slides of Locke-Lewis solution when the growth is poor, and are probably due to some metabolic disturbance.

In order to observe the direct effect of this solution upon the normal cells I treated heart tissue, which was growing well in normal Locke-Lewis solution, with Locke-Lewis 1.5 per cent sodium chloride. In most cases it killed the cells. In some instances normal growths were used, other pieces of which were growing in the hypertonic 1.5 per cent solution, but the shock of this hypertonic solution to the delicate cells, thinly spread out on the cover-slip, was usually so great that they died. I noted especially the fate of two interesting cells (Text-fig. 28) in a 48 hour growth from a 10 day chick heart, which was treated with Locke-Lewis sodium chloride 1.5 per cent solution at 11.15 a.m. The reaction was immediate (Text-fig. 29). Their clear processes were retracted and formed long, fine processes on cell *A*, cell *B* contracting into an irregular mass. The fine processes on *A* were gradually withdrawn until all had disappeared (Text-figs. 30 to 33). *B* showed two distinct kinds of protoplasm—a clear, outer part, resembling the ectoplasm of an ameba and similarly active; and a denser, granular part in the center of the cell. This central mass was more stationary and the clear protoplasm flowed around it, either evenly, or forming blunt processes, which would be withdrawn and then form anew at another place, as described by Cash for cells treated with ether vapor. Later cell *A*

developed the same kind of movement (Text-fig. 34), but at noon was an inactive mass of cytoplasm. By 2.40 p.m. *B* had recovered completely; *A* was still an irregular mass, though the outer cytoplasm was again active (Text-fig. 35). By 4 p.m. (Text-fig. 36) both were active—*B* normal, *A* abnormal. The following day the cells had migrated and changed positions, so that it was impossible to say

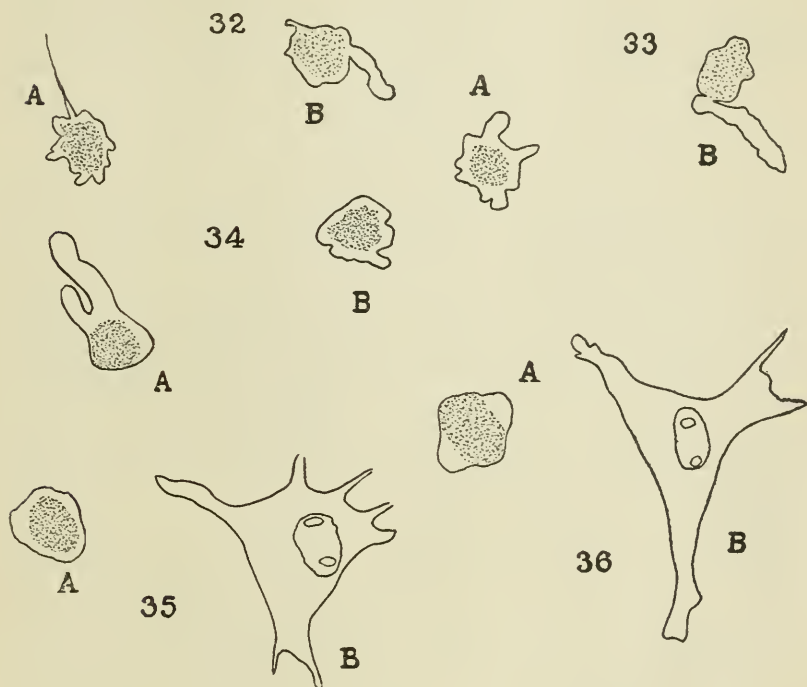


TEXT-FIG. 28. Two normal fibroblasts, *A* and *B*, before treatment with Locke-Lewis solution (1.5 per cent sodium chloride content) at 11.15 a.m.

TEXT-FIGS. 29 to 31. The same cells after treatment. In *A* is shown the formation of long fine processes, which were gradually withdrawn. *B* is an irregular mass showing two kinds of protoplasm, granular and clear, the latter moving like the pseudopodia of *Amœba proteus*.

whether these particular cells had entirely recovered. Half the number of cells on the slide were normal, with stellate forms; hence some of them had been able to withstand the shock. In another case which was followed one cell was apparently killed at once, while a larger

cell with a long process remained active, going through the stage of fine process formation and finally contracting, though the long posterior process remained extended like the posterior end of a *Vorticella*, acting as an anchor.



TEXT-FIGS. 32 to 36. Later stages of the cells shown in Text-figs. 28 to 31. The fine processes of *A* were completely withdrawn and after passing through an amoeboid stage it became an irregular, rather inactive mass of protoplasm. *B* after the amoeboid stage became normal.

Hypertonic Solution, 1.2 Per Cent Sodium Chloride.—Growth in this solution was very good; 32 per cent of the transplants grew, and one lived 15 days without being opened or treated in any way. Growth, however, was much slower than in the controls or in the hypotonic solutions. Often the cells did not begin to migrate until the 2nd or 3rd day, and then slowly, though in growths in which they were going to form a reticulum, they had done so by the 4th

day. Migration was not greater than in the hypotonic solutions but was often abundant, and the appearance of both migratory and reticular formations was that of healthy, normal cells.

When these cells were stained with neutral red and Janus black No. 2 the neutral red granules were found to be present in varying amounts and were arranged around the centriole, as in the controls. Usually there were some long mitochondria, but these varied in size.

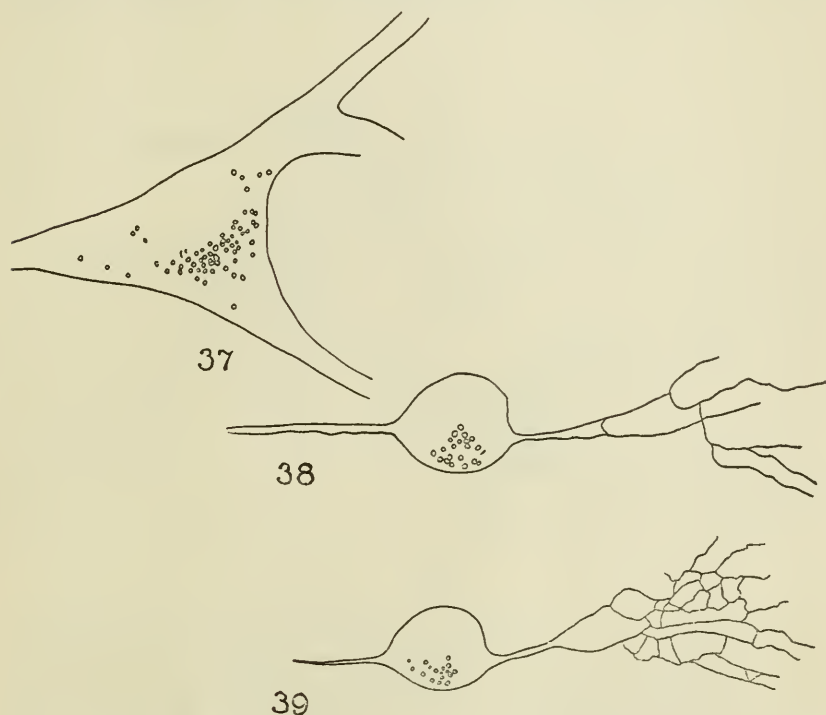
Occasionally the number of migrating cells was very small and these sometimes showed abnormalities. On one slide the cells extended out from the explant like a string of beads, three or four to a string. On another slide, which was later fixed in Zenker's fluid and stained with Ehrlich's hematoxylin, most of the cells were not flat but slightly rotund. The mitochondria were all round, a few neutral red granules were present, and the nucleus in many instances was apparently budding or dividing amitotically, in a manner similar to that shown in some of Macklin's figures. Some cells showed as many as three nuclei of different sizes, though all contained chromatin material. The metabolism of this culture had been seriously deranged, resulting in this change in the nuclear and mitochondrial conditions.

The results following the addition of Locke-Lewis 1.2 per cent sodium chloride solution to normal growths of 1, 2, and 3 days were varied. The reaction was much slower than with the other hypertonic solutions. On three slides of 3 days growth few of the cells showed any contraction after 6 hours of treatment. Many were abnormal and contained a great number of deeply staining neutral red vacuoles. These cells lived and the vacuoles retained the neutral red stain for 1 to 3 days after treatment, or until some of the cells were 8 days old.

On other slides of 2 and 3 days growth interesting changes in the cells were observed. The long, fine, straight processes were also present here. Text-fig. 37 shows a normal cell which at 10.45 a.m. was treated with Locke-Lewis 1.2 per cent sodium chloride solution. At the end of half an hour little change was noted. Within the next hour the cell contracted and a fine network of protoplasmic processes formed at one side, quite a distance from the cell, yet connected with it by a long strand of clear protoplasm (Text-fig. 38).

By 2.10 p.m. (Text-fig. 39) this network had become more intricate, the neutral red granules had almost disappeared, and the cell was practically dead. Later, large clear vacuoles formed in the central mass.

In another cell which was followed the central mass contracted, leaving the clear cytoplasm in a large, thin sheet, stretching out at



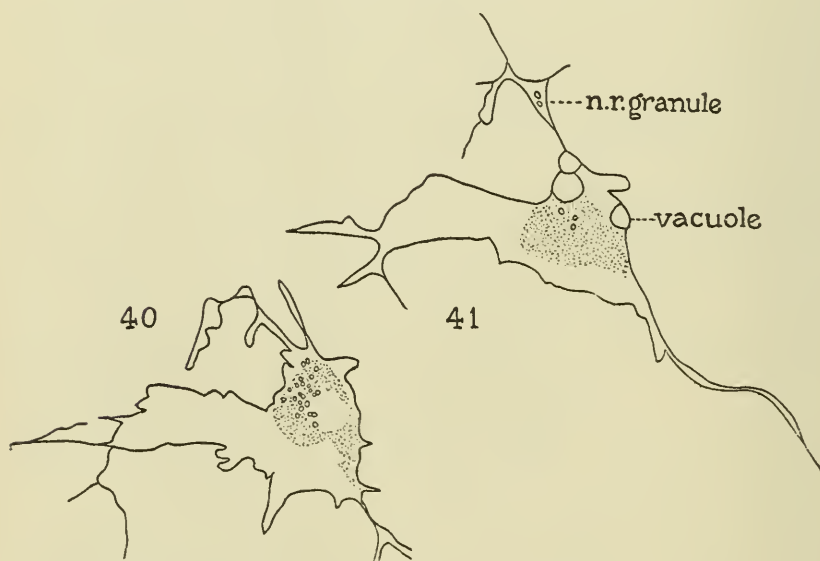
TEXT-FIG. 37. Normal fibroblast of a 7 day chick embryo heart; 3 days growth.

TEXT-FIGS. 38 and 39. The same cell after treatment with Locke-Lewis solution (1.2 per cent sodium chloride content), showing fine protoplasmic network developed from the processes of the cell.

one side of the cell with many slender processes extending in various directions. In Text-fig. 40 can be seen the concentration of neutral red granules in one part of the cell, while in Text-fig. 41 two of the granules have been caught in one of the processes when the central mass began to contract. Here blebs are forming and the large

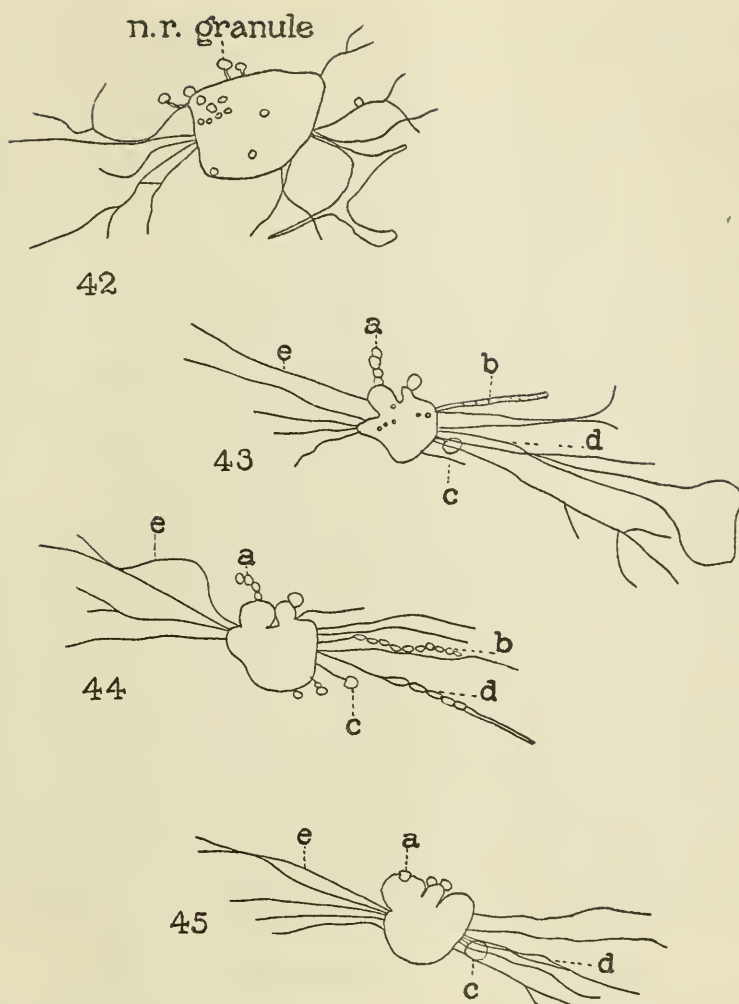
vacuoles indicate that the cell is nearly dead. In Text-fig. 42, from another cell, small neutral red granules are shown alongside of the processes and evidently held to them by fine protoplasmic threads, for they remain stationary though vibrating all the time with Brownian movement.

Still other interesting formations are seen in Text-figs. 43 to 45, from a 2 day growth of a 7 day chick heart. This cell was one of a



TEXT-FIGS. 40 and 41. Fibroblast after treatment with Locke-Lewis solution (1.2 per cent sodium chloride content), showing a contracted clear flat area of cytoplasm where normally there were processes. In Text-fig. 41 two neutral red granules have been caught in one of the processes during contraction of the cytoplasm.

group of three which had been followed after its treatment with Locke-Lewis 1.2 per cent sodium chloride solution at 11 a.m. In Text-fig. 43 at *a* there is a chain of protoplasmic beads which waved back and forth (Text-fig. 44) in the medium. Later, three of these beads broke away and disappeared. At *b* (Text-fig. 43) a slightly thicker process is seen which at 2.40 p.m. showed light and dark areas like a striated muscle. These quickly became constricted into a chain of proto-



TEXT-FIG. 42. Fibroblast treated like that in Text-fig. 40, showing neutral red granules fastened to the cell by slender threads of cytoplasm.

TEXT-FIGS. 43 to 45. Fibroblast treated with Locke-Lewis solution (1.2 per cent sodium chloride content). Many of the fine processes showed movement. The beaded formations at *a* and *b* moved back and forth; *c* was continually oscillating; *d* was moving and *e* waved until it became attached to another fibril (Text-fig. 44); then it moved like an undulating membrane until the cell was dead.

plasmic beads which were free moving and waved about in the surrounding medium (Text-fig. 44). At 3.00 the process broke loose, became entangled with the other thin processes, and disappeared. *c* was a round process of protoplasm attached by a fine thread, which also oscillated back and forth. *d* was apparently attached to a process from a neighboring cell, and in Text-fig. 43 was only a thick process. In Text-fig. 44 it had become beaded, the beads being of different sizes, and was undulating gently. Later (Text-fig. 45) it had become practically straight again, and as the cell was dying the movement gradually lessened. *e* always remained a thin, thread-like process. At first free moving (Text-fig. 43), it later became attached to another process (Text-fig. 44) which was stationary. However, it continued to wave like an undulating membrane until nearly 4 p.m., when motion in the processes ceased and the cell was dead, the color in the neutral red granules having also disappeared.

This long, waving fiber answers the description of those grown by Baitsell in plasma. He believes that they are not outgrowths of the embedded tissue but come from fibrin in the plasma clot. His evidence for this is not complete, as his stains give two different results, one confirmatory of his theory, the other pointing toward the cellular origin of the fibers. In the present experiments there was no plasma and consequently no fibrin. Furthermore, this fibril is directly connected with the cell and remains in active motion only as long as the cell is alive. Its place of origin, the clear, protoplasmic area which contracts to form long, fine processes, is the same as that given by Lewis (1917). She describes the formation of the fibrils of connective tissue from the ectoplasm of the cell.

Summary of Results with Hypertonic Solutions.

1. Hypertonic solutions were made by boiling down Locke-Lewis solution until the sodium chloride content was 1.2, 1.5, and 1.8 per cent respectively.
2. Tissues grew in the first two of these solutions.
3. Tissues did not live so long in these solutions as in normal Locke-Lewis solution and growth was slower.
4. The cells of normal growth were killed by treatment with hypertonic solutions with a sodium chloride content of 1.8 and 1.5 per cent.

5. When treated with hypertonic solutions the cells usually contracted, their thin processes became long and thread-like and were later drawn into the body of the cell.

6. Connective tissue fibrils formed from these thread-like processes. They moved and anastomosed with other fibrils.

7. Neutral red channels formed in many cells.

8. The cytoplasm frequently became alveolar when the death process set in.

9. These three hypertonic solutions showed a definite gradation in their effects on the processes of the fibroblast. In Locke-Lewis solution containing 1.8 per cent sodium chloride the processes contracted rapidly, leaving many thread-like structures in their places. These were quickly withdrawn and the cell soon died. In Locke-Lewis solution containing 1.5 per cent sodium chloride the thread-like processes were frequently formed, but the cells did not all die; some recovered. In Locke-Lewis solution containing 1.2 per cent sodium chloride the processes still formed but more slowly. They also showed motion, which lasted as long as the cell was alive.

Hypertonic versus Hypotonic Solutions.

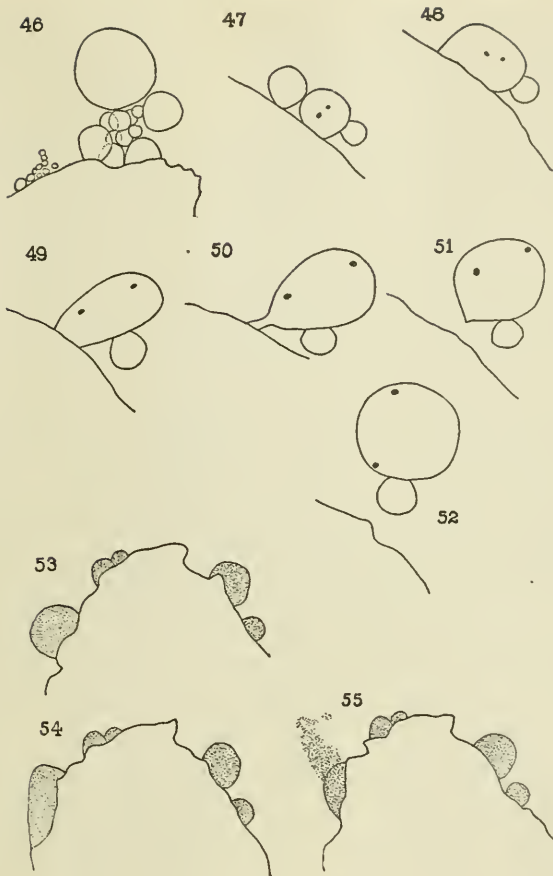
In comparing the growth in hypertonic with that in hypotonic media the following points may be emphasized. In the former the migration is slower than in the latter. In hypertonic solutions the optimum growth is reached on the 3rd day or later, in hypotonic solutions on the 2nd day. The growths live longer in hypertonic solutions but are, as a rule, smaller than in hypotonic solutions. Table I shows this difference of growth in the various media.

TABLE I.

Amount of NaCl in medium.	Total No. of explants.	No. that grew.	No. that died.	Growth.	Greatest No. of days any culture lived in the medium.
<i>per cent</i>				<i>per cent</i>	
1.8	38	0	38	0	0
1.5	130	12	118	9	7
1.2	100	32	68	32	15
0.9	282	188	94	66.6	19
0.54	23	20	3	86.9	7
0.45	202	90	106	44.5	12
0.225	12	0	12	0	0

There seemed to be a possibility of proving whether or not the neutral red granules and vacuoles are the product of metabolism, as Lewis (1919) believes, by an experiment in which parts of the same heart were grown respectively in Locke-Lewis normal solution, Locke-Lewis solution with sodium chloride content of 0.45 per cent, and with 1.2 per cent sodium chloride content, the rate of growth being different in each of these media. Accordingly, pieces of heart from a 7 day chick embryo were planted in the three solutions and incubated for 48 hours. At the end of 24 hours nine of the normal Locke-Lewis cultures, eight of the 0.45 per cent sodium chloride, and two of the 1.2 per cent sodium chloride showed growth. After another 24 hours of incubation eight of the Locke-Lewis, four of the 0.45 per cent sodium chloride, and five of the 1.2 per cent sodium chloride cultures were alive. Here is seen again the slow growth in hypertonic solutions. These cultures were stained with neutral red and Janus black No. 2. Growth in both the Locke-Lewis solution and 1.2 per cent sodium chloride was good, and each showed a normal arrangement of the neutral red granules. In the hypotonic solution growth had been poor. Few cells had migrated, and in these the large neutral red vacuoles were full of deeply stained material. The cells, in their poor condition, were evidently having difficulty in getting rid of the waste products which were accumulating from metabolism.

In working with hypertonic solutions, occasionally with normal Locke-Lewis solution, and in a few instances with hypotonic solutions, I observed an interesting attempt on the part of the tissue to adapt itself to the new medium. Tissues were planted in Locke-Lewis solution containing 1.8 and 1.5 per cent sodium chloride. On the following day transparent, balloon-like structures were noted, varying in size from quite small to very large, which formed along the edges of the culture. Some explants had many of these balloons around the sides, others only a few large ones. Sometimes balloons of all sizes piled up in masses (Text-fig. 46), those at the top being free from the tissue. Upon close observation these structures were seen to begin as small hemispheres rising out of the explant. In time they became almost spherical and increased in size as though something from the tissue was being poured into them. Occa-



TEXT-FIG. 46. Masses of balloons of all sizes piled up at the edge of the heart tissue which had been planted in Locke-Lewis solution of 1.5 per cent sodium chloride content 48 hours before.

TEXT-FIGS. 47 to 52. The formation of a large balloon by the fusion of two small ones (Text-figs. 47 and 48). This large one elongated (Text-fig. 49), formed a stalk (Text-fig. 50), was pinched off (Text-fig. 51), and became free floating (Text-fig. 52).

TEXT-FIG. 53. Granular hills along the edge of a piece of heart planted in Locke-Lewis solution of 1.5 per cent sodium chloride content.

TEXT-FIGS. 54 and 55. One of these granular hills enlarged and discharged its contents into the surrounding medium.

sionally two fused together, as shown in Text-figs. 47 and 48. This particular one continued to increase in size until the pressure became so great that it elongated (Text-fig. 49). A small stalk was formed (Text-fig. 50) which quickly snapped, thus freeing the balloon (Text-fig. 51). It rounded up into a ball within a few seconds (Text-fig. 52) and rolled around over the tissue when the slide was tilted back and forth. It appeared to be taking in culture medium or else flattening out, as it continued to increase in size until one side grew faint, then disappeared, and the balloon went to pieces.

Some cultures were full of these balloons floating around singly or in masses. They were frequently found being given off on the 2nd day, but were not seen to form after that, though those already formed persisted for several days. One slide 11 days old still showed masses of them, but these disappeared on the 12th day. The balloons were clear and transparent, and occasionally contained small particles like cells. There was a distinct surface tension which quickly rounded them up after they were given off. They were also very delicate structures, as evidenced by the fact that when attempts were made to transfer the cover-slip, with the culture in its hanging drop, from the depression slide with the vaseline ring to a straight slide with a large drop of the same hypertonic solution in which the tissue was planted, the balloons nearly always went to pieces, no matter how carefully the cover-slip was lowered onto the new drop of medium. Occasionally this transfer was successful, and on placing the slide under the microscope with dark-field illumination the balloons showed as small, milky white structures not only at the edges, as had appeared with the ordinary illumination, but all over the explant.

There was another type of structure, which I have termed granular hills, that also appeared over the explants. These were more stable and lasted as long as the cultures were kept. They grew in size, sometimes becoming quite large, though they were most frequently seen as small balls or hills along the edges or between the angles of the tissue (Text-fig. 53). They were very finely granular. Sometimes the surface tension would be taxed too much and the granular hill would break open at one place, pouring the fine granules into the surrounding medium (Text-figs. 53 to 55).

Both balloons and granular hills would appear on the same explant. These explants never showed growth or migration of cells, though they would often continue to beat for several days. The structures described above were evidently a means of adjusting the tissue to its new medium. Some days every tissue planted in Locke-Lewis 1.8 and 1.5 per cent sodium chloride solutions would show the balloons or the granular hills, sometimes both. Again, a new medium, made in the same proportions of Locke solution, bouillon, and dextrose, boiled down to the same volume as that used in the preceding experiments, would give few if any of these structures, and also practically no growth. The balloons and granular hills were not the large precipitations, with irregular, angular outlines, which are frequently observed in tissue cultures and which are much more coarsely granular. They may be the same material which Burrows and Neymann state is liberated from the cells when they are removed from their normal habitat to an oxygen-containing plasma or salt solution. These authors state that the substances are "almost transparent, their refraction not very different from that of the original medium, and they accumulate at the surface of the medium to form a membrane." Perhaps the growth in plasma with a different osmotic pressure from Locke-Lewis solution may account for the membrane formation instead of the balloons and granular hills found in my experiments. On the other hand, they may be quite different substances, as Burrows and Neymann state that theirs "are liberated in large amounts from a tissue fragment rich in cells." While the structures described above rarely appeared when there was cell migration, a few times I have found near the explant, after the cells had degenerated, granular hills which had been completely covered over by the cells.

BIBLIOGRAPHY.

- Baitsell, G. A., *J. Exp. Med.*, 1915, xxi, 455.
Burrows, M. T., *Tr. Congr. Am. Phys. and Surg.*, 1913, ix, 77.
Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.
Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiii, 562.
Cash, J. R., *Anat. Rec.*, 1919, xvi, 146.
Cowdry, E. V., *Carnegie Inst. Washington, Pub. No. 271*, 1918, Contributions to embryology, viii, 39.

- Ebeling, A. H., *J. Exp. Med.*, 1914, xx, 130.
Goldschmidt, R., *Proc. Nat. Acad. Sc.*, 1915, i, 220.
Hogue, M. J., *J. Exp. Zool.*, 1917, xxii, 565.
Lambert, R. A., *J. Exp. Med.*, 1914, xix, 398.
Lewis, M. R., *Am. J. Physiol.*, 1915, xxxviii, 153.
Lewis, M. R., *Carnegie Inst. Washington, Pub. No. 226*, 1917, Contributions to embryology, vi, 45.
Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1912, vi, 195.
Lewis, M. R., and Lewis, W. H., *Am. J. Anat.*, 1914-15, xvii, 339.
Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 81.
Macklin, C. C., *Carnegie Inst. Washington, Pub. No. 224*, 1916, Contributions to embryology, iv, 69.
Ruth, E. S., *J. Exp. Med.*, 1911, xiii, 422.
Sundwall, J., *Bull. Hyg. Lab., U. S. P. H., No. 81*, 1912.

EXPLANATION OF PLATES.

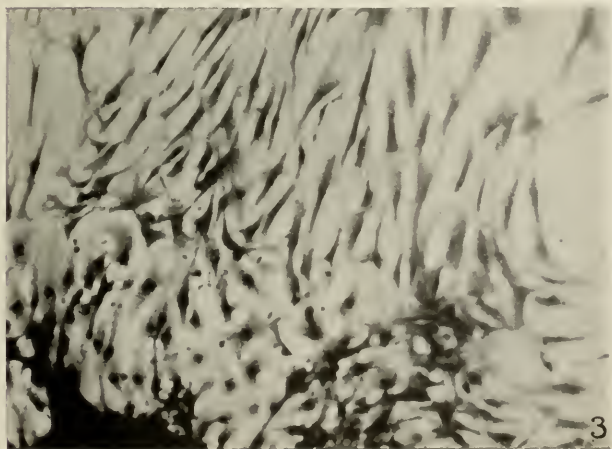
PLATE 56.

FIG. 1. Photomicrograph of normal growth of chick heart tissue, showing fibroblasts at *a* in migration formation, and mesothelial cell in membrane formation at *b*.

FIG. 2. Photomicrograph of 3 day growth of chick heart tissue in hypotonic Locke-Lewis solution (0.45 per cent sodium chloride), showing the early death of cells next to the explant.

FIG. 3. Photomicrograph of a normal 48 hour growth which was treated with hypotonic Locke-Lewis solution (0.45 per cent sodium chloride) and incubated for 24 hours. Some of the cells next to the explant have been killed, but others are migrating out over them.

648^a-



(Hogue: Fibroblasts of embryo chick heart.)

INDEX TO AUTHORS.

A

- Avery, O. T., and Cullen, Glenn E.
Hydrogen ion concentration of
cultures of pneumococci of the
different types in carbohydrate
media, 359
— See DOCHEZ, AVERY, and
LANCEFIELD, 179

B

- Barber, M. A. Antiblastic phe-
nomena in active acquired im-
munity and in natural immunity
to pneumococcus, 589
— A study by the single cell
method of the influence of ho-
mologous antipneumococcic se-
rum on the growth rate of pneu-
mococcus, 569
Brown, Wade H., and Pearce,
Louise. Chemotherapy of try-
panosome and spirochete infec-
tions. Biological series. I. The
toxic action of *N*-phenylglycine-
amide-*p*-arsonic acid, 417
— and —. Biological series. IV.
The action of *N*-phenylglycine-
amide-*p*-arsonic acid upon spiro-
chete infections, 483
— See PEARCE and BROWN,
437, 455

C

- Chase, C. S. See DRAGSTEDT,
DRAGSTEDT, MCCLINTOCK, and
CHASE, 109
Chillingworth, Felix P. See HAR-
RIS and CHILLINGWORTH, 75
Clough, Mildred C. A study of
pneumococci reacting with anti-

pneumococcus sera of Types I, II,
and III, with an observation of
a mutation of one of the strains,
123

- Cullen, Glenn E. See AVERY and
CULLEN, 359

D

- Dochez, A. R., Avery, O. T., and
Lancefield, R. C. Studies on the
biology of streptococcus. I. An-
tigenic relationships between
strains of *Streptococcus hæmolyti-*
cus, 179
Dragstedt, Carl A. See DRAG-
STEDT, DRAGSTEDT, MCCLIN-
TOCK, and CHASE, 109
Dragstedt, Lester R., Dragstedt,
Carl A., McClintock, John T.,
and Chase, C. S. Intestinal ob-
struction. II. A study of the
factors involved in the produc-
tion and absorption of toxic ma-
terials from the intestine, 109

E

- Ebeling, Albert H. A strain of
connective tissue seven years old,
531

F

- Florence, Laura. See SMILLIE, LIT-
TLE, and FLORENCE, 341

H

- Harris, William H., and Chilling-
worth, Felix P. The experi-
mental production in dogs of
emphysema with associated asth-
matic syndrome by means of an
intratracheal ball valve, 75

Harrop, George A., Jr. The oxygen and carbon dioxide content of arterial and of venous blood in normal individuals and in patients with anemia and heart disease, 241

Heidelberger, Michael. See JACOBS and HEIDELBERGER, 411

Hogue, Mary Jane. The effect of hypotonic and hypertonic solutions on fibroblasts of the embryonic chick heart *in vitro*, 617

J

Jacobs, Walter A., and Heidelberger, Michael. Chemotherapy of trypanosome and spirochete infections. Chemical series. I. *N*-phenylglycineamide-*p*-arsonic acid, 411

Jones, F. S. The streptococci of equines, 159

K

Kawamura, K. Studies on organ transplantation. I. Transplantation of the thyroid gland with intact blood supply, 45

— II. Transplantation of the spleen with intact blood supply, 65

Kligler, I. J. Growth accessory substances for pathogenic bacteria in animal tissues, 31

Koser, S. A. See STEVENS and KOSER, 539

L

Lancefield, R. C. See DOCHEZ, AVERY, and LANCEFIELD, 179

Langstroth, Lovell. Blood viscosity. I. Conditions affecting the viscosity of blood after withdrawal from the body, 597

— II. Effect of increased venous pressure, 607

Little, Ralph B. See SMILLIE, LITTLE, and FLORENCE, 341

Lord, Frederick T. The relation of proteolytic enzymes in the pneumonic lung to hydrogen ion concentration. An explanation of resolution, 379

— and Nye, Robert N. The relation of the pneumococcus to hydrogen ion concentration, acid death-point, and dissolution of the organism, 389

Lundsgaard, Christen. Erythrosis, or false cyanosis, 295

— Studies of oxygen in the venous blood. V. Determinations on patients with anemia, 147

— Studies on cyanosis. I. Primary causes of cyanosis, 259

— II. Secondary causes of cyanosis, 271

M

McClintock, John T. See DRAGSTEDT, DRAGSTEDT, MCCLINTOCK, and CHASE, 109

Mills, Ralph G. The pathological changes in the testes in epidemic pneumonia, 505

N

Noguchi, Hideyo. Etiology of yellow fever. IV. The acquired immunity of guinea pigs against *Leptospira icteroides* after the inoculation of blood of yellow fever patients, 1

— V. Properties of blood serum of yellow fever patients in relation to *Leptospira icteroides*, 9

— VI. Cultivation, morphology, virulence, and biological properties of *Leptospira icteroides*, 13

- Noguchi, Hideyo.** VII. Demonstration of *Leptospira icteroides* in the blood, tissues, and urine of yellow fever patients and of animals experimentally infected with the organism, 87
- VIII. Presence of a leptospira in wild animals in Guayaquil and its relation to *Leptospira icterohæmorrhagiæ* and *Leptospira icteroides*, 95
- IX. Mosquitoes in relation to yellow fever, 401
- Nye, Robert N.** See **LORD** and **NYE**, 389

P

- Pearce, Louise, and Brown, Wade H.** Chemotherapy of trypanosome and spirochete infections. Biological series. II. The therapeutic action of *N*-phenylglycineamide-*p*-arsonic acid in experimental trypanosomiasis of mice, rats, and guinea pigs, 437
- and —. Biological series. III. The therapeutic action of *N*-phenylglycineamide-*p*-arsonic acid in experimental trypanosomiasis of rabbits, 455
- See **BROWN** and **PEARCE**, 417, 483

S

- Smillie, Ernest W., Little, Ralph B., and Florence, Laura.** An interpretation of the agglutination re-

- action to *Bacillus abortus* in 75 cases of bovine abortion bacteriologically controlled, 341
- Smith, Theobald.** The bacteriology of bovine abortion, with special reference to acquired immunity, 325
- The etiological relation of spirilla (*Vibrio fetus*) to bovine abortion, 313
- and **Taylor, Marian S.** Some morphological and biological characters of the spirilla (*Vibrio fetus, n.sp.*) associated with disease of the fetal membranes in cattle, 299
- Stadie, William C.** The oxygen of the arterial and venous blood in pneumonia and its relation to cyanosis, 215
- Stevens, F. A., and Koser, S. A.** Streptolysin production in carbohydrate media, 539
- Stillman, Ernest G.** See **WINCHELL** and **STILLMAN**, 497

T

- Taylor, Marian S.** See **SMITH** and **TAYLOR**, 299

W

- Winchell, Agnes I., and Stillman, Ernest G.** The occurrence of *Bacillus influenzae* in the normal throat, 497
- Wollstein, Martha.** Pfeiffer's bacillus and influenza. A serological study, 555

INDEX TO SUBJECTS.

A

Abortion:

- Bovine, bacteriologically controlled, agglutination reaction to *Bacillus abortus* (SMILLIE, LITTLE, and FLORENCE) 341
- , bacteriology (SMITH) 325
- , etiological relation of spirilla (SMITH) 313
- , — of *Vibrio fetus* (SMITH) 313

Abortus:

- Bacillus*, agglutination reaction, in bovine abortion, (SMILLIE, LITTLE, and FLORENCE) 341

Absorption:

- Toxic materials, from intestine (DRAGSTEDT, DRAGSTEDT, McCLINTOCK, and CHASE) 109

Acid:

- Death-point, relation of pneumococcus (LORD and NYE) 389

Agglutination:

- Reaction, to *Bacillus abortus*, in bovine abortion (SMILLIE, LITTLE, and FLORENCE) 341

Anemia:

- Carbon dioxide of arterial blood (HARROP) 241
- — — venous blood (HARROP) 241
- Oxygen of arterial blood (HARROP) 241
- — venous blood (HARROP) 241
- — — — (LUNDSGAARD) 147

Antiblastic:

- Phenomena, in active acquired immunity to pneumococcus (BARBER) 589
- , — natural immunity to pneumococcus (BARBER) 589

Antigen:

- Streptococcus hemolyticus* strains, antigenic relationships (DOCHEZ, AVERY, and LANCEFIELD) 179

Antipneumococcic serum:

- See Serum, pneumococcic.

Artery:

- Blood, arterial, carbon dioxide, in anemia (HARROP) 241
- , —, —, in heart disease (HARROP) 241
- , —, —, in normal individuals (HARROP) 241
- , —, oxygen, in anemia (HARROP) 241
- , —, —, in heart disease (HARROP) 241
- , —, —, in normal individuals (HARROP) 241
- , —, —, in pneumonia (STADIE) 215
- , —, —, relation to cyanosis (STADIE) 215

Asthma:

- Syndrome, associated with emphysema (HARRIS and CHILLINGWORTH) 75

B

Bacillus:

- abortus*, agglutination reaction in bovine abortion (SMILLIE, LITTLE, and FLORENCE) 341

Bacillus—continued:

influenzæ, in normal throat
(WINCHELL and STILLMAN) 497

Pfeiffer's, relation to influenza
(WOLLSTEIN) 555

Bacteria:

Pathogenic, growth accessory
substances for, in animal tis-
sues (KLIGLER) 31

Bacteriology:

Abortion, bovine (SMITH) 325

—, —, bacteriologically con-
trolled, agglutination reac-
tion to *Bacillus abortus* (SMIL-
LIE, LITTLE, and FLORENCE)
341

Biology:

Leptospira icteroides, biologi-
cal properties (NOGUCHI) 13

Spirilla associated with dis-
ease of fetal membranes in
cattle, biological characters
(SMITH and TAYLOR) 299

Streptococcus (DOCHEZ, AVERY,
and LANCEFIELD) 179

Vibrio fetus associated with dis-
ease of fetal membranes in
cattle, biological characters
(SMITH and TAYLOR) 299

Blood:

Arterial, carbon dioxide, in
anemia (HARROP) 241

—, — —, in heart disease
(HARROP) 241

—, — —, in normal individ-
uals (HARROP) 241

—, oxygen, in anemia (HAR-
ROP) 241

—, —, in heart disease (HAR-
ROP) 241

—, —, in normal individuals
(HARROP) 241

Blood—continued:

Arterial, oxygen, in pneumonia
(STADIE) 215

—, —, relation to cyanosis
(STADIE) 215

Leptospira icteroides in, of
animals experimentally in-
fected (NOGUCHI) 87

— — —, of yellow fever pa-
tients (NOGUCHI) 87

Serum of yellow fever patients,
properties, in relation to
Leptospira icteroides (NO-
GUCHI) 9

Supply, intact, transplantation
of spleen with (KAWAMURA)
65

—, —, — of thyroid with
(KAWAMURA) 45

Venous, carbon dioxide, in
anemia (HARROP) 241

—, — —, in heart disease
(HARROP) 241

—, — —, in normal individ-
uals (HARROP) 241

—, oxygen (LUNDGAARD) 147

—, —, in anemia (HARROP)
241

—, —, in heart disease (HAR-
ROP) 241

—, —, in normal individuals
(HARROP) 241

—, —, in pneumonia (STADIE)
215

—, —, relation to cyanosis
(STADIE) 215

Viscosity (LANGSTROTH)
597, 607

—, after withdrawal from body
(LANGSTROTH) 597

Yellow fever patients, im-
munity acquired against *Lep-
tospira icteroides* after inocula-
tion (NOGUCHI) 1

C

Carbohydrate:

- Media, hydrogen ion concentration of pneumococcus cultures in (AVERY and CULLEN) 359
- , streptolysin production in (STEVENS and KOSER) 539

Carbon dioxide:

- Blood, arterial, in anemia (HARROP) 241
- , —, in heart disease (HARROP) 241
- , —, in normal individuals (HARROP) 241
- , venous, in anemia (HARROP) 241
- , —, in heart disease (HARROP) 241
- , —, in normal individuals (HARROP) 241

Cattle:

- Abortion, bacteriologically controlled, agglutination reaction to *Bacillus abortus* (SMILLIE, LITTLE, and FLORENCE) 341
- , bacteriology (SMITH) 325
- , etiological relation of spirilla (SMITH) 313
- , — — of *Vibrio fetus* (SMITH) 313
- Fetal membranes, disease of, biological characters of spirilla associated with (SMITH and TAYLOR) 299
- — —, biological characters of *Vibrio fetus* associated with (SMITH and TAYLOR) 299
- — —, morphology of spirilla associated with (SMITH and TAYLOR) 299
- — —, morphology of *Vibrio fetus* associated with (SMITH and TAYLOR) 299

Cell:

- Pneumococcus, growth rate, influence of homologous anti-pneumococcic serum on, studied by single cell method (BARBER) 569

Chemotherapy:

- Spirochete infections (BROWN and PEARCE) 417, 483
- — (JACOBS and HEIDELBERGER) 411
- — (PEARCE and BROWN) 437, 455
- Trypanosome infections (BROWN and PEARCE) 417, 483
- — (JACOBS and HEIDELBERGER) 411
- — (PEARCE and BROWN) 437, 455

Connective tissue:

See Tissue.

Cultivation:

- Leptospira icteroides* (NOGUCHI) 13

Culture:

- Pneumococcus, hydrogen ion concentration in carbohydrate media (AVERY and CULLEN) 359

Cyanosis:

- (LUNDGAARD) 259, 271
- Causes, primary (LUNDGAARD) 259
- , secondary (LUNDGAARD) 271
- False (LUNDGAARD) 295
- Oxygen of arterial and venous blood in pneumonia, relation of, to (STADIE) 215

D

Death:

- point, acid, relation of pneumococcus (LORD and NYE) 389

Disease:

Fetal membranes, in cattle,
biological characters of spi-
rilla associated with (SMITH
and TAYLOR) 299

—, —, —, biological characters
of *Vibrio fetus* associated with
(SMITH and TAYLOR) 299

—, —, —, morphology of
spirilla associated with
(SMITH and TAYLOR) 299

—, —, —, morphology of
Vibrio fetus associated with
(SMITH and TAYLOR) 299

Heart, carbon dioxide of ar-
terial blood (HARROP) 241

—, —, — of venous blood (HAR-
ROP) 241

—, oxygen of arterial blood
(HARROP) 241

—, —, — venous blood (HAR-
ROP) 241

Dissolution:

Organism, relation of pneumo-
coccus (LORD and NYE) 389

E**Embryo:**

Chick heart, fibroblasts, effect
of hypertonic solutions *in*
vitro (HOGUE) 617

—, —, —, effect of hypotonic
solutions *in vitro* (HOGUE)
617

Emphysema:

Production, by intratracheal
ball valve (HARRIS and CHILL-
INGWORTH) 75

Enzyme:

Proteolytic, in pneumonic lung,
relation to hydrogen ion con-
centration (LORD) 379

Epidemic:

Pneumonia, pathological
changes in testes (MILLS)
505

Erythrosis:

(LUNDGAARD) 295

Etiology:

Spirilla, relation to bovine abor-
tion (SMITH) 313

Vibrio fetus, relation to bovine
abortion (SMITH) 313

Yellow fever (NOGUCHI)
1, 9, 13, 87, 95, 401

F**Fetus:**

Membranes, disease of, in cattle,
biological characters of spi-
rilla associated with (SMITH
and TAYLOR) 299

—, —, —, in cattle, biological
characters of *Vibrio fetus* asso-
ciated with (SMITH and TAY-
LOR) 299

—, —, —, in cattle, morphology
of spirilla associated with
(SMITH and TAYLOR) 299

—, —, —, in cattle, morphology
of *Vibrio fetus* associated with
(SMITH and TAYLOR) 299

Vibrio, biological characters
(SMITH and TAYLOR) 299

—, etiological relation to bo-
vine abortion (SMITH) 313

—, morphology (SMITH and
TAYLOR) 299

Fever:

Yellow. See Yellow fever.

Fibroblast:

Embryonic chick heart, effect
of hypertonic solutions *in*
vitro (HOGUE) 617

—, —, —, effect of hypotonic
solutions *in vitro* (HOGUE)
617

G**Gland:**

Thyroid, transplantation, with
intact blood supply (KAWA-
MURA) 45

Growth:

- Accessory substances for pathogenic bacteria in animal tissues (KLIGLER) 31
- Pneumococcus, rate, influence of homologous antipneumococcal serum, studied by single cell method (BARBER) 569

H**Hæmolyticus:**

- Streptococcus*, strains, antigenic relationships (DOCHEZ, AVERY, and LANCEFIELD) 179

Heart:

- Disease. *See* Disease.
- Embryonic chick, fibroblasts, effect of hypertonic solutions *in vitro* (HOGUE) 617
- — —, effect of hypotonic solutions *in vitro* (HOGUE) 617

Homologous:

- Serum, antipneumococcal, influence on pneumococcus growth rate, studied by single cell method (BARBER) 569

Horse:

- Streptococci* (JONES) 159

Hydrogen ion:

- Concentration, of pneumococcus cultures in carbohydrate media (AVERY and CULLEN) 359
- , relation of pneumococcus (LORD and NYE) 389
- , — — proteolytic enzymes in pneumonic lung (LORD) 379

Hypertonic:

- Solutions, effect on fibroblasts of embryonic chick heart *in vitro* (HOGUE) 617

Hypotonic:

- Solutions, effect on fibroblasts of embryonic chick heart *in vitro* (HOGUE) 617

I**Ictero-hæmorrhagiæ:**

- Leptospira*, relation of leptospira in wild animals (NOGUCHI) 95

Icteroides:

- Leptospira*, acquired immunity against (NOGUCHI) 1
- , biological properties (NOGUCHI) 13
- , cultivation (NOGUCHI) 13
- , in blood of animals experimentally infected (NOGUCHI) 87
- , — — of yellow fever patients (NOGUCHI) 87
- , — tissues of animals experimentally infected (NOGUCHI) 87
- , — — of yellow fever patients (NOGUCHI) 87
- , — urine of animals experimentally infected (NOGUCHI) 87
- , — — of yellow fever patients (NOGUCHI) 87
- , morphology (NOGUCHI) 13
- , properties of blood serum of yellow fever patients in relation to (NOGUCHI) 9
- , relation of leptospira in wild animals (NOGUCHI) 95
- , virulence (NOGUCHI) 13

Immunity:

- Abortion, bovine, acquired (SMITH) 325

Immunity—continued:

Leptospira icteroides (NOGUCHI) 1

Pneumococcus, active acquired, antiblastic phenomena (BARBER) 589

—, natural, antiblastic phenomena (BARBER) 589

In vitro:

Fibroblasts of embryonic chick heart, effect of hypertonic solutions (HOGUE) 617

— of embryonic chick heart, effect of hypotonic solutions (HOGUE) 617

Infection:

Leptospira icteroides in blood of animals experimentally infected (NOGUCHI) 87

— — — tissues of animals experimentally infected (NOGUCHI) 87

— — — urine of animals experimentally infected (NOGUCHI) 87

Spirochete, action of *N*-phenylglycineamide-*p*-arsonic acid (BROWN and PEARCE) 483

—, chemotherapy (BROWN and PEARCE) 417, 483

—, — (JACOBS and HEIDELBERGER) 411

—, — (PEARCE and BROWN) 437, 455

Trypanosome, chemotherapy (BROWN and PEARCE) 417, 483

—, — (JACOBS and HEIDELBERGER) 411

—, — (PEARCE and BROWN) 437, 455

Influenza:

Pfeiffer's bacillus (WOLLSTEIN) 555

Influenzæ:

Bacillus, in normal throat (WINCHELL and STILLMAN) 497

Inoculation:

Yellow fever patients' blood, immunity acquired against *Leptospira icteroides* after (NOGUCHI) 1

Intestine:

Obstruction (DRAGSTEDT, DRAGSTEDT, McCLINTOCK, and CHASE) 109

Toxic materials, absorption (DRAGSTEDT, DRAGSTEDT, McCLINTOCK, and CHASE) 109

— —, production (DRAGSTEDT, DRAGSTEDT, McCLINTOCK, and CHASE) 109

Intratracheal:

Ball valve, producing emphysema (HARRIS and CHILLINGWORTH) 75

L**Leptospira:**

icterohæmorrhagica, relation of leptospira in wild animals (NOGUCHI) 95

icteroides, acquired immunity against (NOGUCHI) 1

—, biological properties (NOGUCHI) 13

—, cultivation (NOGUCHI) 13

—, in blood of animals experimentally infected (NOGUCHI) 87

—, — of yellow fever patients (NOGUCHI) 87

—, — tissues of animals experimentally infected (NOGUCHI) 87

—, — — of yellow fever patients (NOGUCHI) 87

—, — — urine of animals experimentally infected (NOGUCHI) 87

—, — — of yellow fever patients (NOGUCHI) 87

Leptospira—continued:

- icteroides*, morphology (NOGUCHI) 13
 - , properties of blood serum of yellow fever patients in relation to (NOGUCHI) 9
 - , relation of leptospira in wild animals (NOGUCHI) 95
 - , virulence (NOGUCHI) 13
 - Presence in wild animals (NOGUCHI) 95
 - Relation of leptospira in wild animals to *Leptospira icterohæmorrhagiae* (NOGUCHI) 95
 - — — in wild animals to *Leptospira icteroides* (NOGUCHI) 95
- Lung:**
- Pneumonic, proteolytic enzymes in, relation to hydrogen ion concentration (LORD) 379

M

Medium:

- Carbohydrate, hydrogen ion concentration of pneumococcus cultures in (AVERY and CULLEN) 359
- , streptolysin production in (STEVENS and KOSER) 539

Membrane:

- Fetal, disease of, in cattle, biological properties of spirilla associated with (SMITH and TAYLOR) 299
- , — —, in cattle, biological properties of *Vibrio fetus* associated with (SMITH and TAYLOR) 299
- , — —, in cattle, morphology of spirilla associated with (SMITH and TAYLOR) 299

Membrane—continued:

- Fetal, disease of, in cattle, morphology of *Vibrio fetus* associated with (SMITH and TAYLOR) 299

Method:

- Cell, single, influence of homologous antipneumococcic serum on pneumococcus growth rate studied by (BARBER) 569

Morphology:

- Leptospira icteroides* (NOGUCHI) 13
- Spirilla associated with disease of fetal membranes in cattle (SMITH and TAYLOR) 299
- Vibrio fetus* associated with disease of fetal membranes in cattle (SMITH and TAYLOR) 299

Mosquito:

- Yellow fever, relation (NOGUCHI) 401

Mutation:

- Pneumococcus strain (CLOUGH) 123

O

Obstruction:

- Intestinal (DRAGSTEDT, DRAGSTEDT, McCLINTOCK, and CHASE) 109

Organ:

- Transplantation (KAWAMURA) 45, 65

Organism:

- Dissolution, relation of pneumococcus (LORD and NYE) 389
- Leptospira icteroides*, in blood of animals experimentally infected (NOGUCHI) 87
- —, in tissues of animals experimentally infected (NOGUCHI) 87
- —, in urine of animals experimentally infected (NOGUCHI) 87

Oxygen:

- Blood, arterial, in anemia (HARROP) 241
- , —, in heart disease (HARROP) 241
- , —, in normal individuals (HARROP) 241
- , —, in pneumonia (STADIE) 215
- , —, relation to cyanosis (STADIE) 215
- , venous (LUNDGAARD) 147
- , —, in anemia (HARROP) 241
- , —, in heart disease (HARROP) 241
- , —, in normal individuals (HARROP) 241
- , —, in pneumonia (STADIE) 215
- , —, relation to cyanosis (STADIE) 215

P**Pathogenicity:**

- Bacteria, pathogenic, growth accessory substances for, in animal tissues (KLIGLER) 31

Pathology:

- Testes, in epidemic pneumonia (MILLS) 505

Pfeiffer's bacillus:

See Bacillus.

N-Phenylglycineamide-*p*-arsonic acid:

- (JACOBS and HEIDELBERGER) 411
- Spirochete infections, action on, of (BROWN and PEARCE) 483
- Toxic action (BROWN and PEARCE) 417
- Trypanosomiasis, therapeutic action on, of (PEARCE and BROWN) 437, 455

Pneumococcus:

- Acid death-point, relation (LORD and NYE) 389
- Cultures, hydrogen ion concentration in carbohydrate media (AVERY and CULLEN) 359
- Dissolution of organism, relation (LORD and NYE) 389
- Growth rate, influence of homologous antipneumococcic serum on, studied by single cell method (BARBER) 569
- Hydrogen ion concentration, relation (LORD and NYE) 389
- Immunity, active acquired, antiblestic phenomena (BARBER) 589
- , natural, antiblestic phenomena (BARBER) 589
- Reaction with Type I, II, and III antipneumococcus sera (CLOUGH) 123
- Pneumonia:**
 - Epidemic, pathological changes in testes (MILLS) 505
 - Lung, proteolytic enzymes in, relation to hydrogen ion concentration (LORD) 379
 - Oxygen of arterial blood (STADIE) 215
 - — venous blood (STADIE) 215

Pressure:

- Venous, increased, effect on blood viscosity (LANGSTROTH) 607

Proteolysis:

- Enzymes, proteolytic, in pneumonic lung, relation to hydrogen ion concentration (LORD) 379

R

Reaction:

- Agglutination, to *Bacillus abortus*, in bovine abortion (SMILLIE, LITTLE, and FLORENCE) 341
- Pneumococcus, with Type I, II, and III antipneumococcus sera (CLOUGH) 123

Resolution:

- (LORD) 379

S

Serology:

- Influenza (WOLLSTEIN) 555

Serum:

- Pneumococcic, homologous, influence on pneumococcus growth rate, studied by single cell method (BARBER) 569

- , of Types I, II, and III, pneumococci reacting with (CLOUGH) 123

- Yellow fever patients, properties, in relation to *Leptospira icteroides* (NOGUCHI) 9

Solution:

- Hypertonic, effect on fibroblasts of embryonic chick heart *in vitro* (HOGUE) 617

- Hypotonic, effect on fibroblasts of embryonic chick heart *in vitro* (HOGUE) 617

Spirillum:

- Etiological relation to bovine abortion (SMITH) 313

- Fetal membranes, disease of, in cattle, biological characters of spirilla associated with (SMITH and TAYLOR) 299

- — —, in cattle, morphology of spirilla associated with (SMITH and TAYLOR) 299

Spirochete:

- Infections, action of *N*-phenylglycineamide-*p*-arsonic acid (BROWN and PEARCE) 483

- , chemotherapy (BROWN and PEARCE) 417, 483

- , — (JACOBS and HEIDELBERGER) 411

- , — (PEARCE and BROWN) 437, 455

Spleen:

- Transplantation, with intact blood supply (KAWAMURA) 65

Strain:

- Connective tissue, old (EBELING) 531

- Pneumococcus, mutation (CLOUGH) 123

- Streptococcus haemolyticus*, antigenic relationships (DOCHEZ, AVERY, and LANCEFIELD) 179

Streptococcus:

- Biology (DOCHEZ, AVERY, and LANCEFIELD) 179

- haemolyticus*, strains, antigenic relationships (DOCHEZ, AVERY, and LANCEFIELD) 179

- Horse (JONES) 159

Streptolysin:

- Production in carbohydrate media (STEVENS and KOSER) 539

Syndrome:

- Asthmatic, associated with emphysema (HARRIS and CHILLINGWORTH) 75

T

Testicle:

- Pathology, in epidemic pneumonia (MILLS) 505

Therapy:

N-Phenylglycineamide-*p*-arsonic acid, in experimental trypanosomiasis (PEARCE and BROWN) 437, 455

Throat:

Bacillus influenzae in normal (WINCHELL and STILLMAN) 497

Thyroid:

Transplantation, with intact blood supply (KAWAMURA) 45

Tissue:

Connective, old strain (EBELING) 531

Growth accessory substances for pathogenic bacteria in animal (KLIGLER) 31

Leptospira icteroides in, of animals experimentally infected (NOGUCHI) 87

— — —, of yellow fever patients (NOGUCHI) 87

Toxicity:

Materials, toxic, from intestine, absorption (DRAGSTEDT, DRAGSTEDT, McCLINTOCK, and CHASE) 109

—, —, — —, production (DRAGSTEDT, DRAGSTEDT, McCLINTOCK, and CHASE) 109

N-Phenylglycineamide-*p*-arsonic acid (BROWN and PEARCE) 417

Transplantation:

Organ (KAWAMURA) 45, 65

Spleen, with intact blood supply (KAWAMURA) 65

Thyroid, with intact blood supply (KAWAMURA) 45

Trypanosome:

Infections, chemotherapy (BROWN and PEARCE) 417, 483

—, — (JACOBS and HEIDELBERGER) 411

Trypanosome—continued:

Infections, chemotherapy (PEARCE and BROWN) 437, 455

Trypanosomiasis:

N-Phenylglycineamide-*p*-arsonic acid, therapeutic action in experimental (PEARCE and BROWN) 437, 455

Type:

Pneumococci of different types, hydrogen ion concentration in carbohydrate media (AVERY and CULLEN) 359

Sera, antipneumococcus, of Types I, II, and III, pneumococci reacting with (CLOUGH) 123

U**Urine:**

Leptospira icteroides in, of animals experimentally infected (NOGUCHI) 87

— — —, of yellow fever patients (NOGUCHI) 87

V**Valve:**

Ball, intratracheal, producing emphysema (HARRIS and CHILLINGWORTH) 75

Vein:

Blood, venous, carbon dioxide, in anemia (HARROP) 241

—, —, — —, in heart disease (HARROP) 241

—, —, — —, in normal individuals (HARROP) 241

—, —, oxygen (LUNDGAARD) 147

—, —, —, in anemia (HARROP) 241

—, —, —, in heart disease (HARROP) 241

Vein—continued:

- Blood, venous, oxygen, in normal individuals (HARROP) 241
- , —, —, in pneumonia (STADIE) 215
- , —, —, relation to cyanosis (STADIE) 215
- Pressure, increased, effect on blood viscosity (LANGSTROTH) 607

Vibrio:

- fetus*, biological characters (SMITH and TAYLOR) 299
- , etiological relation to bovine abortion (SMITH) 313
- , morphology (SMITH and TAYLOR) 299

Virulence:

- Leptospira icteroides* (NOGUCHI) 13

Viscosity:

- Blood (LANGSTROTH) 597, 607
- , after withdrawal from body (LANGSTROTH) 597

Y**Yellow fever:**

- Blood of patients, *Leptospira icteroides* in (NOGUCHI) 87
- Etiology (NOGUCHI) 1, 9, 13, 87, 95, 401
- Immunity against *Leptospira icteroides* acquired after inoculation of blood of patients (NOGUCHI) 1
- Mosquitoes, relation (NOGUCHI) 401
- Serum of patients, properties, in relation to *Leptospira icteroides* (NOGUCHI) 9
- Tissues of patients, *Leptospira icteroides* in (NOGUCHI) 87
- Urine of patients, *Leptospira icteroides* in (NOGUCHI) 87

R

11

J68

v.30

cop.3

Biological

& Medical

Serials

1919

The Journal of
experimental medicine

PLEASE DO NOT REMOVE
CARDS OR SLIPS FROM THIS POCKET

UNIVERSITY OF TORONTO LIBRARY
